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Abstract: Equine influenza viruses are a major cause of respiratory disease in horses worldwide and undergo antigenic drift. Several outbreaks of equine influenza occurred worldwide during 2010-2012, including in vaccinated animals, highlighting the importance of surveillance and virus characterisation. Virus isolates were characterised from more than 20 outbreaks over a 3-year period, including strains from the UK, Dubai, Germany and the USA. The haemagglutinin-1 (HA1) sequence of all isolates was determined and compared with OIE-recommended vaccine strains. Viruses from Florida clades 1 and 2 showed continued divergence from each other compared with 2009 isolates. The antigenic inter-relationships among viruses were determined using a haemagglutination-inhibition (HI) assay with ferret antisera and visualised using antigenic cartography. All European isolates belonged to Florida Clade 2, all those from the USA belonged to Florida Clade 1. Two subpopulations of Clade 2 viruses were isolated, with either substitution A144V or I179V. Isolates from Dubai, obtained from horses shipped from Uruguay, belonged to Florida Clade 1 and were similar to viruses isolated in the USA the previous year. The neuraminidase (NA) sequence of representative strains from 2007 and 2009 to 2012 was also determined and compared with that of earlier isolates dating back to 1963. Multiple changes were observed at the amino acid level and clear distinctions could be made between viruses belonging to Florida Clade 1 and Clade 2.

Revision note VETMIC-D-13-8580

Authors' response to reviewer comments:

Reviewer #1: This manuscript is a composite of two topics - one that relates to an enhanced surveillance program in the UK for equine influenza virus (and presumably any other pathogens) and one that characterizes newer EIV isolates from various parts of the world. I am not convinced that the two parts stand together very well. If the surveillance program had resulted in the detection of novel EIV isolates that would have been missed in a passive surveillance model, then I could be convinced the two elements should be in the same manuscript. However, there is no evidence that the enhanced program added anything to the detection of EIV in the period presented. More samples were received but no connection was made to the isolates analyzed. A "free" surveillance program is nice to have, but for the bulk of the world, it is not a program that is feasible with the economic resources available.

We agree with the reviewer that there are two topics here. However, we also believe that they are closely linked and that the increase in diagnostic sample submission should be sufficient justification to include the strategies used to encourage practitioners to submit nasal swabs. In response, we have therefore deleted surveillance data from the results & discussion, but retained the approaches used in the method section, for reference purposes.

The scheme only relates to equine influenza as the financial support we have from the HBLB only covers 'flu. This is considered the major threat to horse racing and breeding in the UK, due to its rapid spread in an unprotected population.

The 'free' surveillance scheme allows us to characterise samples sent from other countries as well as the UK. We believe that for countries where funding could potentially be sought, this scheme provides examples of low-cost methods that can be used to improve surveillance & sample submission, such as Twitter. We fully accept that this isn't possible in all countries.

The isolate characterization data is certainly of value in monitoring the changes in EIV. What is disappointing is that all of the work done so far does not seem able to predict when a new vaccine will be needed. The antigenic mapping and serology tests tell us that changes are occurring and where in the HA and NA, but little about the efficacy of the current vaccines. Field data still seems most reliable. One wonders whether making ferret antisera in the future is worth the effort.

The vaccine strain selection process for EIV, as described in previous publications, is loosely based on that for human influenza – i.e. four or more changes in HA1 amino acid sequence, affecting two or more antigenic sites (mapped for human H3) coupled with an 8-fold difference in HI titre using ferret sera. Unfortunately a lot more work needs to be done on antigenic characterisation of EIV as the antigenic sites have not yet been defined and we are still a long way off being able to predict

when vaccine breakdown is likely to occur. An added complication is that, unlike human influenza vaccine production, the infrastructure does not exist to update equine vaccines within a year and the process typically takes 3-5 years. This makes it even more important to have an effective 'early warning' system. The current system relies on monitoring changes in the field and attempting to predict when antigenically significant changes have occurred. Waiting for vaccine breakdown to occur in the field would not be ideal as the purpose of the OIE strain recommendations is to try and prevent this.

Regarding the efficacy of current vaccines - as part of our work as an OIE reference laboratory, we deliberately avoid 'naming and shaming' commercial products as it has proven more effective to maintain a good dialogue with all the vaccine manufacturers to encourage the regular updating of strains. Politically, we also have to remain impartial. There is current evidence of vaccine breakdown in several countries, but we are not at liberty to report this in detail as the data does not belong to us, they are reported in outline by the OIE. The best we can do is to publish up to date sequence and antigenic data to make it clear that the Florida clade1 & clade2 viruses continue to diverge from vaccine strains in current use.

Ferret sera have proven extremely useful at demonstrating antigenic changes in influenza viruses from humans and horses, equine sera are notoriously cross-reactive and extremely expensive to produce. There are also Home Office & ethical implications for using companion animals for this process. Unpublished data from our group indicates that equine sera show very similar patterns of recognition compared to ferrets, but at a lower fold difference e.g. equine sera will show a 2-fold difference when ferrets may reach 4-16.

Specific comments:
1 Line 35. This sentence sort of reflects the awkward transition from the surveillance discussion to real data. My recommendation is to eliminate the surveillance material and focus on the isolates. Perhaps a letter to Vet Record for the surveillance would be more appropriate.

Surveillance material removed from abstract (and results section)

2. Line 103. "considerable changes" Somewhat subjective designation - when does "few" become considerable?

'multiple' substituted for 'considerable'.

3. Line 138. Reference for "maximal sensitivity"?

OIE terrestrial codes manual chapter 2.5.7 has been added to the references

4. Line 148. Just curious what per cent of isolates are only detected on the 4th blind pass.

We don't 'blind' pass, allantoic fluid is always tested at each passage by HA assay to keep the number of passages to a minimum. None of our isolates since 2006 have come up at P4 if negative at P3; if our samples are negative at P2 they come up at P3 very rarely, but we routinely passage to P3 to be sure.

5. Line 167. I will admit to being a poor statistician, but my impression was that geometric means are used on data sets when the range of serology values differ widely (4 -4048). The HI titers probably vary by two-fold and a simple arithmetic average might be more correct (96 vs 91).

Advice was originally taken from WHO experts who use GMT for human influenza virus HI tables. Titres for a given serum/antigen combination can vary by 2 fold in either direction, as the assay is based on serial two-fold dilutions, leading to a 4-fold variance in HI values which rarely reach above 512. Our resident statistician agrees that GMT is the most appropriate method for this type of log_2 data.

6. Line 230. How many isolates came from the surveillance program?

Surveillance data has been deleted from the manuscript.

7. Table 1 and Table X (no number and no reference in text but assume to be list of isolates from outside UK). I would recommend putting these in "Supplementary data" as the information is of limited value other than the Genbank reference number.

We disagree, for those working in the field of EIV, the outbreak information in Table 1 is of value. Table 2 (outside the UK) has been transferred to supplementary data as requested as there is minimal information in this other than accession numbers..

8. Figure 1. As SA/4/03 is a vaccine strain, it should be included in this figure.

SA/4/03 is highlighted in bold in the figure but may be easy to miss for those unfamiliar with the H3N8 tree. The figure legend has been altered to make this clear.

9. Figure 1 Legend. Line 654. Should there be a reference for the reassortant strains? Also Figure 4.

The reassortant strains were identified in this manuscript, by sequencing NA, so there is no other reference available. The legends for figures 2 and 4 have been altered to make this clear.

10. Lines 300-303. Data not shown?

Text adjusted, to remove reference to this data.

11. Figure 3. Lines 667-670. Should the Figure have the a,b,c label and if so, the legend does not match the figure - a and b are switched. Figure legend altered to remove reference to a/b/c.

Figure legend has been altered to remove reference to a, b and c.

12. Figure 2. Make clear distinction in figure between clade 1 and clade 2. Should not have to refer to other figures to note where the clade switch occurs. Same issue with Figure 5.

Clade 1 and clade 2 labels have been added to the HA alignments (figure 2). Figure 5 has been labelled to show all the sublineages present (pre-divergence, European, American, Florida clades 1 & 2)

13. Figure 7, Line 705. Someone forgot to put A/equine/Richmond in the Fig. Also, the larger spheres and larger squares are not evident in the copy of the Figure that downloaded. Bit more attention to detail in the figure would be nice.

An error was made by us and we submitted the wrong version of this figure with the original manuscript. The correct version has been inserted, Richmond/07 is shown in black, as described in the figure legend. Larger spheres are highlighted in a different colour (turquoise) so should be easy to distinguish. The sera indicated by larger squares have now been marked in heavy black and the remaining sera in grey to improve the contrast and should be evident in the figure provided.

14. Lines 409-410. Statement is a bit vague about whether isolates actually came from the enhanced surveillance program.

We don't understand the comment, this line refers to the amino acid differences between Florida clade 1 and 2 in 2003, which do not relate to the surveillance programme.

15. We seem to be left with the conclusion that none of the data were helpful in defining the next vaccine recommendations or perhaps the current vaccine is adequate for the viruses currently circulating.

Text has been adjusted in the conclusion to clarify that current OIE recommendations are considered adequate.

Development of a surveillance scheme for equine influenza in the UK and characterisation of viruses isolated in Europe, Dubai and the USA from 2010-2012

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Abstract

Equine influenza viruses are a major cause of respiratory disease in horses worldwide and undergo antigenic drift. Several outbreaks of equine influenza occurred worldwide during 2010-2012, including in vaccinated animals, highlighting the importance of surveillance and virus characterisation. ~~To encourage submission of samples for isolation of current field strains, a sentinel practice scheme of over 180 equine practices was established in the UK.~~ Virus isolates were characterised~~obtained~~ from more than 20 outbreaks over a 3-year period, including strains~~. Isolates were also submitted~~ from the UK, Dubai, Germany and the USA. The haemagglutinin-1 (HA1) sequence of all isolates was determined and compared with OIE-recommended vaccine strains. Viruses from Florida clades 1 and 2 showed continued divergence from each other compared with 2009 isolates. The antigenic inter-relationships among viruses were determined using a haemagglutination-inhibition (HI) assay with ferret antisera and visualised using antigenic cartography. All European isolates belonged to Florida Clade 2, all those from the USA ~~and Dubai~~ belonged to Florida Clade 1. Two subpopulations of Clade 2 viruses were isolated, with either substitution A144V or I179V. Isolates from Dubai, obtained from horses shipped from Uruguay, belonged to Florida Clade 1 and were similar to viruses isolated in the USA the previous year. The neuraminidase (NA) sequence of representative strains from 2007 and 2009 to 2012 was also determined and compared with that of earlier isolates dating back to 1963. Multiple changes were observed at the amino acid level and clear distinctions could be made between viruses belonging to Florida Clade 1 and Clade 2.

Keywords: equine influenza virus, H3N8, surveillance, antigenic cartography, neuraminidase

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Introduction

Equine influenza virus (EIV) is a major cause of respiratory disease in horses and spreads rapidly between naïve animals. Although rarely fatal in otherwise healthy horses, EIV can cause severe disruption to the racing and breeding industries. It can also cause more severe clinical signs in animals with concurrent disease, such as hyperadrenocorticism, or in those under physiological stress.

Influenza A viruses are subtyped according to their surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). HA mediates virus entry, by binding to sialic acid receptors on the host cell surface and mediating fusion of viral and host membranes (Skehel & Wiley, 2000). NA is involved in virus release from infected cells by cleaving sialic acid, it may also play a role in virus entry by allowing the virus to penetrate the mucus layer of the respiratory tract (Seto & Rott, 1966; Matrosovich et al, 2004).

Two subtypes of influenza are known to have infected horses, H3N8 and H7N7. Equine H7N7 was first isolated in 1956, equine H3N8 emerged in 1963 and spread globally over the following two years. Between 1963 and the late 1970s both subtypes co-circulated in horses and reassortment occurred between them, indicating the occurrence of mixed infections (Ito et al, 1999). There have been isolated reports of seroconversions to H7N7 in unvaccinated animals, however virus of this subtype has not been isolated since 1979 and has been considered extinct for 20 years (Madić et al., 1996; Webster, 1993). During the 1980s the H3N8 subtype diverged into 2 sub-lineages, Eurasian and American (Daly et al., 1996). The American lineage has since been divided further into the Kentucky, South American and Florida sublineages (Lai et al., 2001). More recently, the Florida sub-lineage has diverged into two clades, based on HA sequence and antigenic differences (Bryant et al., 2009; Lewis et al., 2011). Between 2006 and 2009, Florida clade 2 was seen predominantly in Europe with

77 occasional isolation of clade 1 strains in the UK and Ireland; in North America, recent
78 isolates have all belonged to Clade 1 (Gagnon et al., 2007; Damiani et al., 2008; Bryant et al.,
79 2011; Gildea et al., 2012).

80 Both clades have caused large outbreaks of equine influenza in the last 10 years. Examples
81 include the UK in 2003 (Clade 2), Japan and Australia in 2007 (Clade 1), India in 2009
82 (Clade 2), Mongolia and China from 2008 to 2009 (Clade 2) and most recently in several
83 countries in South America during 2012 (Clade 1) (Newton et al., 2006; Callinan, 2008; Ito et
84 al., 2008; Yamanaka et al., 2008; Virmani et al., 2010; OIE-WAHID interface).

85 Vaccination is an effective method of control for equine influenza, providing protection by
86 the induction of antibodies to viral surface glycoproteins, particularly HA. The role of
87 antibodies to NA is unclear for EIV, however antibodies to human influenza NA are thought
88 to contribute to protective immunity and neutralising titres correlate with reduced virus
89 shedding in small animal models [Murphy et al., 1972; Brett & Johansson, 2005]. Like other
90 influenza viruses, EIV undergoes antigenic drift and is able to evade antibody responses to
91 divergent strains (Yates et al., 2000). Vaccine strains for equine influenza therefore need to
92 be updated regularly and a formal process of vaccine strain selection is in place, overseen by
93 the World Organisation for Animal Health (OIE). Genetic, antigenic and epidemiological
94 data are considered prior to recommending changes to vaccine strains; current OIE
95 recommendations are to include a representative of both Florida clade 1 and clade 2 viruses.
96 To date, genetic and antigenic characterisation for selection of vaccine strains has focussed
97 solely on the HA glycoprotein and antigenic drift in NA has been largely ignored.

98 Our aim was to improve the monitoring of field strains of EIV. Here we describe the
99 establishment of a surveillance programme for EIV in the UK, to encourage the submission
100 of equine nasal swab samples. We present the HA1 sequences and antigenic characterisation

of recent field strains from the UK, Dubai, Germany and USA and compare them with current OIE vaccine strain recommendations. We show that the Florida cladeClade 1 and cladeClade 2 viruses have diverged further since the OIE recommendation to include both in commercial vaccines. We also show that multipleconsiderable changes have occurred within the NA gene segment of equine influenza H3N8 viruses since 1963.

Methods

Sentinel practice scheme

An invitation letter was sent to 60 veterinary practices with equine practitioners within the UK. Further practices were recruited to the scheme by invitation following submission of samples to the diagnostic laboratories at the Animal Health Trust (AHT). Participants were offered free diagnostic testing for samples from equids with suspected influenza, either nasal swabs or paired serum samples. A telephone helpline and dedicated website (www.equiflunet.org.uk) were also made available. Sampling packs were sent to each contributing veterinary practice, including submission forms, virus transport medium and swabs. Newsletters were also distributed to keep participants informed of relevant information. All positive diagnoses were followed up by telephone contact to collect epidemiological data, including vaccination histories of affected animals.

Diagnostic testing for presence of EIV

Nasopharyngeal swabs were taken from horses showing signs of acute respiratory disease, or close contacts of affected animals. Swabs were placed in sterile tubes containing 5 mL virus transport medium (PBS containing 200 U/mL streptomycin, 150 U/mL penicillin, 5µg/mL fungizone (Gibco) and 600 µg/mL tryptone phosphate broth). All equine nasopharyngeal swabs sent to the AHT from 2010 to 2011 were then assayed by an in house nucleoprotein-ELISA as described previously (Cook et al, 1988). Briefly, plates were coated with rabbit polyclonal serum against A/equine/Sussex/89 (H3N8), nasal swab extract was added to the plates and incubated for up to one hour. After washing, bound influenza antigen was detected by incubation with a monoclonal antibody to equine influenza virus nucleoprotein followed by anti-mouse peroxidase conjugated secondary antibody (Dako) and colorimetric assay. All

samples confirmed positive by NP ELISA were then subjected to RNA extraction and egg isolation as described below. Nasopharyngeal swabs sent to the AHT during 2012 were assayed by qRT-PCR as described previously (Bryant et al, 2010), using SensiFAST Probe Hi-ROX Onestep kit (Bioline) and a StepOne Plus qPCR instrument (Applied Biosystems). North American samples were tested by qRT-PCR (Lu et al, 2009) or by the Directigen™ Flu A test kit (BD, New Jersey, USA) according to the manufacturer's directions.

Diagnostic serology assay

The presence of antibodies to EIV in serum samples was determined by haemagglutination inhibition (HI) assay, using 1% chicken erythrocytes according to World Health Organisation standard procedures (WHO). For maximal sensitivity, sera were tested against Tween-treated viruses including A/equine/Prague/56 [H7N7], A/equine/Miami/63 [H3N8] and A/equine/Newmarket/2/93 [H3N8] (OIE 2012).~~.~~ Where possible, paired serum samples taken 14 days apart were analysed.

Virus isolation in eggs

Virus isolation was attempted from all swabs that were diagnosed positive by NP ELISA or qRT-PCR. Briefly, 0.1 ml each swab extract was inoculated into the allantoic cavity of two 10-day-old fertilized hen's eggs at neat, 10^{-1} and 10^{-2} dilutions and incubated at 34°C. Three days later, eggs were chilled at 4ºC overnight, allantoic fluid harvested and a haemaglutination (HA) assay performed to assess virus growth. Swabs giving a negative result after one round in eggs were passaged up to 3 times, checking for growth at each step to minimise the final number of passages. Working stocks were generated from virus isolates by inoculation of eggs at a 10^{-4} dilution, to reduce the risk of generating defective interfering particles. For most EIV strains, this was equivalent to approximately 10 to 100 EID_{50} per egg.

153 Antigenic characterisation by HI assay

154 Ferret antisera were raised against representative strains by intranasal instillation of 0.1 ml
155 EIV per nostril, equivalent to a final dose of $2x10^6$ EID_{50}. Sera were collected three weeks
156 post-infection and stored at -20°C. Prior to use, 300 µl each antiserum was incubated with
157 600 µl 0.38% potassium periodate for 15 min at room temperature, then 300 µl 3% glycerol-
158 PBS was added and the mixture incubated for a further 15 min at room temperature before
159 heat-inactivation at 56°C for 30 min. Equine antisera from AHT archives were raised by
160 aerosol challenge of Welsh mountain ponies and collected at least two weeks post-infection,
161 those against American strains were supplied by the Gluck Equine Research Center and
162 generated in the same manner from mixed-breed ponies. Equine sera were treated as
163 described for ferret. HI assays were carried out using a 96-well format, according to standard
164 procedures (WHO). Briefly, viruses were diluted to 4 HA units in a volume of 25 µl and
165 back-titrated to ensure accuracy. Two-fold serial dilutions of each ferret serum were prepared
166 in PBS and incubated with virus for 30-60 min at room temperature then 50 µl of 1% chicken
167 erythrocytes added. Samples were incubated at 4°C for 45 min prior to scoring. HI assays
168 were carried out at least twice and geometric means calculated. Isolates from different years
169 were grouped in separate batches, but each batch was run against the full panel of reference
170 antigens to allow comparison of data. Quantitative analyses of the ferret HI data were
171 performed using antigenic cartography, as described previously for human H3N2 and equine
172 H3N8 viruses (Smith et al., 2004; Lewis et al, 2011).

173 RNA extraction, RT PCR and sequencing

174 RNA was extracted from all ELISA-positive swabs using a QIAamp viral RNA mini kit
175 (Qiagen) according to the manufacturer's instructions. A 2-step PCR protocol was used,
176 comprising a reverse transcription (RT) step using uni-12 primer, 5'-AGCGAAAGCAGG-3'

and SuperScript II Reverse Transciptase from Invitrogen followed by PCR with either HA1-specific primers 5'-GCGAGCGAAAGCAGGGG-3' and 3'-GCGGATTTGCTTTTCTGGTAC-5' or NA-specific primers 5'-AGCAAAAGCAGGAGTTT-3' and 3'-AACTCCTTGTTTCTACT-5'. The PCR protocol consisted of an initial denaturation step of 92^{o}C for 5 minutes followed by 30 cycles of 95^{o}C for 1 minute, 50^{o}C for 1.5 minutes and 72^{o}C for 5.5 minutes. PCR products were separated by gel electrophoresis using a 1% agarose gel and visualised with GelRed (Biotium). PCR products were purified using kits supplied by Qiagen or Bioline, according to manufacturer's recommendations. PCR products were sequenced using ABI BigDye® Terminator v3.1 (Applied Biosystems) according to manufacturer's instructions on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Sequence analysis and Phylogenetic trees

Nucleotide sequences were visualized and edited using Seqman II version 5.03 (DNAstar Inc) and BioEdit (Ibis Pharmaceuticals Inc.). All sequences were deposited with Genbank. Nucleotide sequences were aligned to representative reference panels for HA1 or NA obtained from Genbank using ClustalW2 (EMBL-EBI). Derived amino acid sequences were aligned against representative strains from each sublineage of EIV, including pre-divergence, Eurasian, American (Kentucky), Florida Clades 1 and 2. Maximum-likelihood (ML) phylogenetic trees for the nucleotide sequences encoding HA1 (1009 nt) and NA (1410 nt) were created using PhyML version 3 (Guindon et al., 2009). Amino acid alignments were generated separately for isolates compared against A/equine/Richmond/1/07 using BioEdit version 7.0.5.3 (Ibis Pharmaceuticals Inc.).

Results

~~Establishment of sentinel practice scheme & rapid notification systems~~

~~To encourage the submission of equine nasal swab and paired blood samples from suspected cases of equine influenza, a sentinel practice scheme was established at the Animal Health Trust. The aim was to recruit equine practitioners that were likely to see unvaccinated horses in the UK, rather than Thoroughbred and other high level competition horses, as these were the most likely to provide samples for virus isolation. The initial approach of 60 equine practices, which excluded those known to have clients primarily with Thoroughbreds, recruited 23 participants to the scheme. Over a period of 6 years, over 150 additional practices were recruited by several approaches: follow up from submission of samples to the AHT diagnostic service, distribution of newsletters or leaflets, recommendation by other participants or equine specialists, contact via the Equiflunet website (www.equiflunet.org.uk), or unsolicited requests. At the close of 2012, 188 veterinary practices were registered to the scheme and 84 submitted samples during that year. The number of veterinary surgeons from each practice contributing to the scheme ranged from 1 to 10. To improve communication of influenza outbreaks to equine practitioners, the 'Tell-Tail' rapid notification system was developed in collaboration with Merial Animal Health. Any positive diagnoses were submitted to Merial by email and a text message sent to all veterinary surgeons who had registered for the scheme. An 'Equiflunet' Twitter account (@equiflunet) was also established for instant notification of outbreaks of equine influenza and messages were captured by a feed system linked to the Equiflunet website.~~

Outbreaks and sample submission: 2010 to 2012

Equine influenza outbreaks within the UK that were diagnosed from either nasal swabs or paired serum samples between January 2010 and December 2012 are summarised in Table 1. Twenty one counties were affected in England, Wales and Scotland with multiple outbreaks in some areas. ~~The number of samples submitted per year via the sentinel practice scheme increased during this period, from 119 in 2010 to 251 in 2012. During 2010, a total of 894 samples were sent to the AHT for diagnosis by NP ELISA, 389 samples were sent in for serological testing by haemagglutination inhibition (HI) assay and 2 samples were sent for single radial haemolysis assay (SRH) assay. Of the samples sent, only 61 were submitted via the sentinel practice scheme for NP ELISA and 58 for HI testing. Of a total of 16 positive nasal swabs, virus was successfully isolated from 14; 2 horses were diagnosed positive by HI assay.~~

~~During 2011, 650 nasal swab samples were submitted, 202 serum samples for HI assay and 3 for SRH testing. Of those tested, 132 samples were submitted through the sentinel practice scheme, comprising 12 blood or serum samples for HI assay and 120 nasal swab samples for ELISA. Ten nasal swabs were diagnosed positive by ELISA, submitted from 7 premises in the UK. Of these, 8 viruses were isolated successfully in eggs. HA1 nucleotide sequence was obtained from 9 samples, either directly from the swab, egg isolated virus or both. Two horses were diagnosed positive by HI assay of paired sera during 2011. None of the samples submitted during 2011 came from horses known to have been vaccinated recently.~~

~~During 2012, very few samples were diagnosed positive from the UK until November. During the year, 427 nasal swab samples were submitted and 200 sera. Of these, 113 nasal swabs and 138 blood samples were sent in via the sentinel practice scheme; 12 were positive by NP ELISA, qPCR or HI assay. Seven viruses were isolated in eggs at first or second~~

passage. In addition to samples submitted from the UK, 17 virus isolates obtained between 2010 and 2012 were received from the Gluck Equine Research Center, OIE reference laboratory for equine influenza in the USA (supplementary data). Outbreaks in the USA were reported from 6 states in 2010, 10 states in 2011 and 17 in 2012, many of which were described by Pusterla et al. (2011) and subsequent work by that group. During 2011 and 2012, there were also outbreaks of equine influenza reported in Germany, including cases in vaccinated animals. One isolate from 2012 was submitted to the AHT by the OIE reference laboratory for Germany, for antigenic characterisation. Three virus isolates were also submitted from the Central Veterinary Laboratories, Dubai, following an outbreak in a quarantine facility. The source of infection was a group of endurance horses transported from Uruguay to Dubai, consistent with reports to the OIE of extensive outbreaks of equine influenza affecting around 2,500 horses in Uruguay during 2012. The endurance horses had received a primary course of two doses of vaccine, according to the manufacturer's recommendations.

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Genetic analyses - HA

HA1 sequences were obtained from all virus isolates, plus one swab sample from which virus could not be isolated (East Renfrewshire/1/11). For most isolates, sequence was also determined directly from the nasal swab extract. In all instances, the HA1 nucleotide sequence obtained from both egg isolate and swab extract was identical, suggesting that no significant selection had occurred during egg passage. Phylogenetic analysis was carried out for the recent isolates against a panel of 130 equine H3N8 HA1 nucleotide sequences from GenBank. The resulting analysis grouped the viruses into 5 well-defined clusters, corresponding to the Pre-divergent, Eurasian, American and Florida clade 1 and 2 sublineages (Figure 1). These clusters were each supported by high bootstrap values, ranging

from 97 to 100 after 100 replicates. All viruses isolated in the UK between 2010 and 2012 belonged to the Florida clade 2 sublineage, all those characterised from the USA were of the Florida clade 1 sublineage. The strains from Dubai were most similar to clade 1 isolates from Kentucky 2011. Derived amino acid sequences were aligned against the current OIE recommended strain for Florida clade 2 (A/equine/Richmond/1/07), shown in Figure 2. In the alignment, each strain is representative of multiple isolates with identical HA1 sequences.

The Florida clade 2 viruses appeared to have 3 consistent amino acid substitutions compared to Richmond/1/07, two of which were P103L and V112I (Figure 2). A further substitution, E291D, was observed between Richmond/1/07 and all of the other strains described here; this was shared by all the clade 2 viruses isolated in the UK during 2007 (Bryant et al, 2009). Two different sub-populations were isolated in the UK during the period studied here, those with an additional change at position 144 and those with a substitution at 179, which was also observed in recent isolates from France and Germany. Amongst the American clade 1 viruses, three amino acid substitutions were unique to 2010 isolates from California: D31N, T163I and I230V. There were 5 consistent amino acid changes between the current clade 1 OIE-recommended strain (South Africa/4/03) and isolates from 2009 onwards: G7D, R62K, D104N, A138S and V223I. The HA2 sequence for some of the isolates was also determined which revealed amino acid substitutions between the recommended vaccine strains and the most recent Florida clade 1 and 2 viruses (data not shown). These substitutions in HA2 included N169S and L187M between Richmond/1/07 and clade 2 isolates, and I198V between South Africa/4/03 and the clade 1 isolates. There were five amino acid substitutions in HA2 between the most recent clade 2 and clade 1 isolates (T43A, E50G, N169S, L187M and I198V).

The conserved amino acid substitutions between the Florida clade 1 and Florida clade 2 viruses were mapped to the structure of the trimeric HA molecule, using A/duck/Ukraine/68 [H3N8] PDB 1MQL (Ha et al, 2003), shown in Figure 3. Four differences mapped to the top of the molecule, close to the receptor binding site, with a further 12 mapping on the surface of the molecule. For comparison, the differences between the clade 2 viruses and the OIE recommended strain Richmond/1/07 and the clade 1 viruses and the recommended strain South Africa/4/03 are also shown. The clade 1 versus clade 2 comparison shows multiple differences between strains, whereas the OIE-recommended strain for each clade shows only one or two differences on the top of the HA molecule. ~~The clade 2 versus American vaccine strains comparison shows a large number of differences between strains, including a ring of substitutions at the top of the molecule around the receptor binding site and multiple changes further down the molecule.~~

Genetic analyses – NA

To investigate the level of variation in NA amongst circulating strains, the nucleotide sequence of segment 6 was determined for 19 strains isolated between 2010 and 2012 from the UK, USA, Germany and Dubai, as well as representative clade 1 and 2 isolates from 2007 and 2009. Phylogenetic analyses were carried out as described for HA, using a panel of 99 sequences from Genbank from 1963 to 2011 (Figure 4). The topology of the NA tree was similar to that of HA and separation of viruses belonging to Florida clades 1 and 2 was well-supported with a bootstrap value of 98%. Major clades also correlated with significant country-wide outbreaks in 1979, 1989, 2003 and 2007. Three recent Florida clade 1 isolates, (Dorset/09, Lanarkshire/09 and Yorkshire/3/09) had an NA segment that was more similar to those of the clade 2 viruses and one Florida clade 2 isolate (Perthshire/1/09) had an NA more

317 similar to those of clade 1 viruses, indicating that reassortment had taken place between clade
318 1 and clade 2. These isolates are highlighted in Figure 4.

319

320 Derived amino acid sequences were aligned against representative strains from four major
321 clades identified by phylogenetic analysis and are shown in Figure 5. Multiple amino acid
322 substitutions were observed between sublineages with signature substitutions readily
323 identified for the current Florida clade 1 and clade 2 viruses compared with the older
324 American strains. There were 16 amino acid substitutions between the most recent UK
325 isolates from clade 1 and clade 2. The majority of changes occurred within the first 80
326 residues, including the membrane anchor sequence and stalk region of NA; these are not
327 included in the protein structure solved for various subtypes of NA. For the purpose of
328 structure mapping, the amino acid numbering of the predicted ectodomain was adjusted to
329 correspond to that present in the H5N1 and H3N8 NA protein structure database files 2HTY
330 and 2HT5. Multiple changes occurred on the surface of NA, shown mapped on the tetrameric
331 structure of H5N1 (Figure 6), affecting both the distal and proximal surfaces of the molecule.
332 An additional substitution V147I within the 150 loop was observed in equine viruses from
333 the Japanese and Australian outbreak in 2007.

334 Antigenic characterisation

335 Low passage virus isolates were characterised by HI assay using post infection ferret antisera
336 raised against eight representative EIV strains and the homologous reference strains. Sera
337 included those raised against representatives from relevant sublineages [American
338 (Kentucky), Florida clade 1, Florida clade 2], current UK vaccine strains and OIE
339 recommended strains, as indicated in Table 2. All virus isolates raised low titres against the

European sera (data not shown), as expected from their genetic characterisation. Ferret antisera against the American Kentucky lineage vaccine strains Newmarket/1/93 and Kentucky/98 recognised the Florida clade 2 isolates from 2010 to 2012, but gave a slightly lower titre than against homologous strains. They typically showed a 2-fold difference for Kentucky/98 and 2- to 4-fold lower titre for Newmarket/1/93. These sera recognised the Florida clade 1 viruses poorly, with most strains showing a 16- to 64- fold reduction against Newmarket/1/93 and an 8- to 16-fold lower titre against Kentucky/98 compared to homologous antigen. Ferret antisera were raised against the early representative of the Florida clade 2 viruses, Kentucky/97, which has sequence similarity to the older American strains, and outbreak strains Newmarket/5/03 and Richmond/1/07 (the current OIE-recommended clade 2 strain). These sera all recognised the clade 2 isolates from 2010 to 2012 and in most instances, to at least the same level as their respective homologous strains. None of the sera raised the maximum titre against their homologous strains; this was particularly noticeable for Kentucky/97 (Table 2). Most of the clade 2 field isolates gave a 2- to 4-fold higher titre than Kentucky/97. All three of the antisera raised to clade 2 viruses gave lower titres against the clade 1 isolates from 2010 to 2012 than the clade 2 strains, reflecting the genetic differences between the two groups. Three sera were raised against clade 1 strains, including South Africa/4/03, one of the current OIE recommended strains for vaccines. The reciprocal pattern was seen with these sera, all sera recognised the clade 1 field isolates with high titres but with lower titres against the clade 2 strains. In both directions, 8- to 16-fold differences in titre were common suggesting antigenic divergence between the two clades.

Antigenic cartography

The antigenic relationships between 44 equine influenza A(H3N8) viruses, including the recent isolates described above and a reference panel of representative Florida clade 1 and

clade 2 strains are shown in Figure 7. The viruses grouped into two distinct antigenic clusters, with all the Florida clade 2 isolates falling into the blue cluster and all the clade 1 isolates grouping together in the red cluster. This is consistent with our previous findings, showing that the two phylogenetic clades were antigenically distinct (Lewis et al, 2011). The current recommended vaccine strains, A/equine/Richmond/1/07 and A/equine/South Africa/4/03, for either clade were located within their respective clusters and the antigenic distance between each isolate and the representative strain did not exceed 2.1 antigenic units, equivalent to a 4-fold difference in HI titre. When we measured the antigenic distances from ferret sera raised to current and previous OIE-recommended strains for the Clade 2 cluster, we found that on average, currently circulating strains were 1.5 antigenic units from the A/equine/Richmond/1/2007 serum, but on average 2 antigenic units from a previously recommended vaccine strain, A/equine/Newmarket/1/1993 serum.

HI of strains against Richmond and South Africa equine sera

To determine whether the antigenic differences between strains belonging to the American, Florida clade~~Clade~~ 1 and Florida clade~~Clade~~ 2 sublineages could be distinguished by equine sera, HI assays were carried out against a panel of post-infection equine sera for a representative selection of strains from the three groups (Table 3). Compared with ferret sera, the titres were lower for equine sera, with homologous titres only reaching 128 rather than up to 1024. The two American sera showed lower titres against viruses from both of the Florida sublineages than the American strains; the antiserum raised against the older strain Kentucky/91 raised even lower titres than Kentucky/99. Although differences were subtle, these sera appeared to recognise the clade~~Clade~~ 1 strains better than clade~~Clade~~ 2, in contrast to ferret sera raised against similar strains (Table 2).

Of the sera raised against more recent viruses, both sera from Richmond/1/07-infected ponies recognised all three groups of viruses, with no more than a 2-fold difference in titre between field isolates and Richmond/1/07. The results for the South Africa clade~~Clade~~ 1 sera were slightly different, with the highest titres raised against homologous or very closely related clade~~Clade~~ 1 viruses. Differences in HI titre within this group of viruses were less than 2-fold compared with the homologous titre for South Africa/4/03. However, the clade~~Clade~~ 1 sera consistently raised lower HI titres against both American and clade~~Clade~~ 2 viruses, giving differences of 2- to 4-fold compared with the South Africa/4/03 strain.

Discussion

Equine influenza viruses belonging to the H3N8 subtype are thought to have crossed the species barrier from birds in the early 1960s and subsequently spread worldwide. They have continued to circulate since then, causing widespread outbreaks in naïve populations, such as South Africa in 2003 and Australia in 2007, but also occasionally in vaccinated horses. Extensive outbreaks in Europe in highly vaccinated horses in the late 1980s demonstrated that circulating equine influenza viruses had undergone significant antigenic drift from the strains included in vaccines and highlighted the need for effective surveillance programmes. ~~Here we describe recent developments to the surveillance scheme established in the UK, based upon a network of sentinel practices from which equine samples were sent in for free diagnostic testing. A variety of media were used to communicate information with equine practitioners, including telephone, post, email, website and social media (Twitter, Tell Tail). At least 10% of the practices submitted samples each year between 2006 and 2011 and 46% in 2012; live virus was recovered successfully every year, making antigenic characterisation of strains possible.~~

In 2003, two large scale epidemics of equine influenza in the UK and South Africa led to the division of the Florida sublineage of equine influenza into two clades, 1 and 2. The original difference was based upon two amino acid substitutions within HA1 at positions 78 and 159, which were sufficient to cause an antigenic difference that was recognisable by ferret sera (OIE, 2008). Since then, the two clades have diverged further and we show here that there are now 9 consistent amino acid differences between viruses from the different groups isolated in 2012 with a further 2 substitutions between subsets of the clade 2 viruses. Additional variants arose during this period as a result of reassortment between clade 1 and clade 2 viruses, which led to new combinations of HA and NA.

In 2010, the OIE recommended that vaccine manufacturers should include a representative of both Florida clade 1 and Florida clade 2 viruses (OIE 2010). Surveillance data reported here confirm that viruses from both clades continued to circulate, with viruses of Florida clade 1 likely to be the cause of multiple large-scale outbreaks in South America. Clade 2 viruses predominated in Europe, causing outbreaks in the UK, France, Ireland and Germany. Surveillance data therefore support inclusion of both clades in vaccines for horses that travel between continents.

Antigenic characterisation of the strains reported here indicated that representatives of the Florida clades 1 and 2 could be distinguished from each other readily, with differences ranging between 2- and 16-fold by HI. These results were comparable to those seen with viruses isolated in 2009 (Bryant et al., 2011), suggesting that the ferret model does not distinguish between viruses with HA1 molecules containing the most recent amino acid differences. These substitutions were primarily conservative changes, the notable exception being the P103L substitution seen in recent clade 2 viruses. However, this position is buried within the HA molecule and is not likely to affect the binding of ferret antisera. Similarly, ferret sera raised against Kentucky lineage vaccine strains from the 1990s continued to recognise Florida clade 2 viruses. However, there was typically a difference of 4-fold between the titres against the most recent clade 2 viruses from 2011 and 2012 compared with the homologous American reference strains Newmarket/1/93 and Kentucky/98, whereas some of those from 2010 showed only a 2-fold difference. Caution should be observed in interpretation of cross-reactive titres, as ferret sera to Newmarket/1/93 vaccine strain cross-reacted well against the Newmarket/5/03 outbreak strain from the 2003 epidemic in the UK, however commercial vaccines containing this strain failed to protect against the outbreak strain (Newton et al., 2006). Cross-reaction was therefore a poor indicator of cross protection in this instance. Clade 1 ferret sera cross-reacted well against other members of the group and

447 also recognised clade 2 viruses, but to a lower level. Interestingly, equine sera raised against
448 Richmond/1/07 reacted well against viruses belonging to all 3 sublineages whereas South
449 Africa/4/03 did not react as well against the clade 2 viruses. This result suggests that
450 Richmond/1/07 could make a good vaccine strain for protecting against both clade 1 and
451 clade 2 viruses, with the caveat outlined above that cross-reaction does not necessarily
452 correlate with cross-protection. Ideally, equine sera would be used for antigenic
453 characterisation of equine influenza virus strains, however these sera are often broadly cross-
454 reactive making them insensitive to antigenic differences between strains. In our experience,
455 the HI titres are often low and strains that give a 4- or 8-fold difference with ferret sera may
456 only raise a 2-fold difference using equine sera, making interpretation difficult.

457 To date, vaccine strain selection for EIV has focussed solely on HA genetic and antigenic
458 differences and there is a lack of sequence data available for NA for recent isolates from
459 Florida clade 2. Comparison of amino acid sequences shown here indicated that NA had
460 undergone considerable divergence since 1963 and outbreak strains from 1979, 1989, 2003
461 and 2007 had multiple amino acid changes compared with previous sublineages. Recent
462 Florida clade 1 and clade 2 viruses differed by 16 or more amino acids and it was clear that
463 reassortment had taken place in strains isolated in the UK. Multiple changes occurred within
464 the stalk region of NA and so could not be mapped on the structure of the tetrameric head
465 region of the NA; however changes also occurred on both the upper and lower surfaces of the
466 head region, including regions close to the active site and within the C-terminal region where
467 human sera have been shown to recognise epitopes in the NA of H5N1 viruses (Khurana et
468 al., 2011). Also of interest was the V147I substitution observed in isolates from the 2007
469 outbreaks in Japan and Australia. All other H3N8 viruses of avian or equine origin, with NA
470 sequences currently available on Genbank, had valine at this position. The exceptions were
471 the H3N8 canine isolates from the USA, which had isoleucine (data not shown). This

position is equivalent to V149 of the N1 structure and falls within the 150 loop of NA (Russell et al, 2006), an important region for NA activity (Lin et al. 2010). The conformation of this loop may affect the size of the adjacent 150 cavity, which potentially differs between group 1 and group 2 NAs.

Further work is required to determine the role of antibodies to NA in immunity in the horse but they may contribute to vaccine efficiency and immune selection (Johansson et al., 1998).

Conclusion

Equine influenza Florida sublineage clade 1 and 2 viruses continued to cause outbreaks worldwide between 2010 and 2012. Clade 2 predominated in Europe while clade 1 was isolated in North and South America. Sequence analysis of NA revealed that reassortment had occurred between the two clades and some virus isolates from 2009 had new combinations of HA and NA. The two sublineages have diverged further since 2009 and can be distinguished readily by antigenic analysis. Current OIE vaccine strain recommendations for representatives of Florida clade 1 and clade 2 remain adequate, based on antigenic differences determined by HI.

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503

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624

Tables

Table 1. Outbreaks of EIV in the UK 2010 to 2012.

Footnote: NP~~FC1- Florida sublineage clade 1 (A/eq/South Africa/4/03 like), FC2- Florida sublineage clade 2 (A/eq/Newmarket/5/03 like),~~ ELISA – nucleoprotein enzyme~~Enzyme~~ linked immunosorbent assay~~Immunosorbent Assay~~, qPCR – quantitative polymerase chain reaction, HI – haemagglutination inhibition assay, HA~~Directigen BD diagnostics, Immunoassay Optical ImmunoAssay Kit, Viva Diagnostika, HA1. Binax Now – Binax, HA1~~ Acc. – Haemagglutinin ~~1~~ accession numbers, NA Acc. – Neuraminidase accession numbers.

Table 2. HI titres of EIV strains using ferret sera.

Footnote: The lineage of new isolates is indicated on the left and ordered by isolation date. Homologous titres are shown in bold, titres for sera against strains from the same sublineage are highlighted in grey boxes. New~~N~~/1/93 – A/eq/Newmarket/1/93, ~~N/2/93 – A/eq/Newmarket/2/93,~~ Ken/97 – A/eq/Kentucky/97, Ken/98 – A/eq/Kentucky/98, New~~N~~/5/03 – A/eq/Newmarket/5/03, SA/4/03 – A/eq/South Africa/4/03, Rich~~Ric~~/1/07 – A/eq/Richmond/1/07, Lin/1/07 – A/eq/Lincolnshire/1/07, Dor/09 – A/eq~~equine~~/Dorset/09. Am – American lineage, FC1 – Florida sublineage clade 1, FC2 - Florida sublineage clade 2.

Table 3. HI titres of EIV strains using post-infection equine sera.

Footnote: The lineage of strains tested is indicated on the left. Titres~~titres~~ for sera against strains from the same sublineage are highlighted in grey boxes, homologous titres are

shown in bold. Ken/91 – A/eq~~equine~~/Kentucky/91, Ken/99 - A/eq~~equine~~/Kentucky/99, Rich/07 - A/eq~~equine~~/Richmond/1/07, Ken/4/07 – A/eq/Kentucky/4/07, SA/4/03 – A/eq/South Africa/4/03. For Rich/07 and SA/4/03 antisera, numbers 1 and 2 relate to serum samples from pony 1 and pony 2 respectively. Am – American lineage, FC1 – Florida sublineage clade 1, FC2 - Florida sublineage clade 2.

Supplementary data

Table S1 Virus isolates from outside the UK: Genbank accession numbers. FC1 – Florida sublineage clade 1, FC2 - Florida sublineage clade 2.

Figure captions

Figure 1

HA Phylogenetic Tree

Phylogenetic analysis of HA1 nucleotide sequences encoded by EIV. A maximum likelihood tree was created using PhyML version 3. Bootstrap values obtained after 100 replicates are shown at major nodes. Amino acid substitutions are indicated at branch points, or in brackets. Phylogenetic groups are shown by continuous bars on the right, as indicated. Accession numbers for the sequences reported in this manuscript are listed in Table 1. Sequences are coloured by date of isolation for the years 2010 (green), 2011 (red) and 2012 (blue) with the older isolates in black. Representative OIE-recommended vaccine strains A/eq/Richmond/1/07 and A/eq/South Africa/4/03 are shown in bold. Reassortant strains containing HA from one Florida clade and NA from the other, identified in this manuscript, are highlighted in~~Reassortant strains are highlighted~~ yellow.

Figure 2

Alignment of HA1 amino acid sequences.

Derived amino acid differences in HA1 observed between representative field strains from Florida clade 2 and clade 1 isolated during 2010-2012 compared to A/eq~~equine~~/Richmond/1/07 (top). The sublineage of isolates is indicated on the left and ordered by isolation date. Residues are numbered from 1 to 329 starting with the serine residue downstream of the predicted signal peptide. Amino acid identity to A/eq~~equine~~/Richmond/1/07 is shown with a dot. Examples of strains from 2009 are included to allow comparison with Bryant et al (2011).

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Figure 3

HA structures.

Location of the amino acid differences on the A/duck/Ukraine/1/63 H3 HA structure (Ha et al, 2003) between OIE recommended Florida clade 1 strain A/eq/South Africa/4/03 and 2010-2012 clade 1 isolates,~~(a)~~ OIE recommended Florida clade 2 strain A/eq~~equine~~/Richmond/1/07 and 2010-2012 clade 2 isolates and~~(b) OIE recommended Florida clade 1 strain A/equine/South Africa/4/03 and 2010-2012 clade 1 isolates (c)~~ 2010-2012 isolates from Florida clade 1 and clade 2. Established changes are shown in red, those that appeared in one year only are shown in blue. HA1 residues 112, 179, 230 and 251 are buried. HA1 residue 7 and HA2 residues 187 and 198 are not shown.

Figure 4

NA Phylogenetic Tree

Phylogenetic analysis of NA nucleotide sequences encoded by EIV. A maximum likelihood tree was created using PhyML version 3. Bootstrap values obtained after 100 replicates are shown at major nodes. Amino acid substitutions are indicated at branch points, or in brackets. Phylogenetic groups are shown by continuous bars on the right, as indicated. Accession numbers for the sequences reported in this manuscript are listed in Table 1. Sequences are coloured by date of isolation for the years 2010 (green), 2011 (red) and 2012 (blue) with the older isolates in black. Representative OIE-recommended vaccine strains A/eq/Richmond/1/07 and A/eq/Ohio/03 (A/eq/South Africa/4/03-like) are shown in bold. Reassortant strains containing NA from one Florida clade and HA from the other, identified in this manuscript, are highlighted in yellow.

Figure 5

Alignment of NA amino acid sequences.

Derived amino acid differences in NA observed between representative strains from Pre-divergent, American, Eurasian, Florida clade 12 and Florida clade 21 sublineages compared to A/eq/Miami/63 (top). The sublineage of isolates is indicated on the left and ordered by isolation date. Residues are numbered from 1 to 470 starting with the methionine residue at the start of the predicted signal peptide. The numbering has not been adjusted to correspond to N1 or N2 numbering. Amino acid identity to A/eq/Miami/63 is shown with a dot.

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Figure 6

NA structure

Location of the amino acid differences on the H5N1 NA structure (Russell et al., 2006) between ~~(a)~~ OIE recommended Florida clade 2 strain A/eq~~equine~~/Richmond/1/07 and 2010-2012 clade 2 isolates, ~~(b)~~ OIE recommended Florida clade 1 strain A/eq~~equine~~/South Africa/4/03 and 2010-2012 clade 1 isolates, as well as ~~(c)~~ 2010-2012 isolates from Florida clade 1 and clade 2. Established changes are shown in red, those that appeared in one year only are shown in blue.

Figure 7

Antigenic cartography

Antigenic map showing the relationships between virus strains isolated between 2010 and 2012. Virus strains are shown as spheres, the positions of sera are shown as open boxes. The OIE-recommended vaccine strains are indicated in black (A/eq~~equine~~/South Africa/4/2003 &

A/eqequine/Richmond/1/2007) and their corresponding sera as larger squares; representative strains from clade 1 (A/eq/Lincolnshire/1equine/Lincoln/2007, A/eqequine/Dorset/200909), clade 2 (A/eqequine/Kentucky/199797, A/eqequine/Newmarket/5/2003) and current commercial vaccine strains(A/eqequine/Newmarket/1/199393, A/eqequine/Kentucky/199898) are highlighted as larger spheres in turquoise. The scale bar indicates one antigenic unit, equivalent to a 2-fold difference in HI titre.

Development of a surveillance scheme for equine influenza in the UK and characterisation of viruses isolated in Europe, Dubai and the USA from 2010-2012

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Abstract

Equine influenza viruses are a major cause of respiratory disease in horses worldwide and undergo antigenic drift. Several outbreaks of equine influenza occurred worldwide during 2010-2012, including in vaccinated animals, highlighting the importance of surveillance and virus characterisation. Virus isolates were characterised from more than 20 outbreaks over a 3-year period, including strains from the UK, Dubai, Germany and the USA. The haemagglutinin-1 (HA1) sequence of all isolates was determined and compared with OIE-recommended vaccine strains. Viruses from Florida clades 1 and 2 showed continued divergence from each other compared with 2009 isolates. The antigenic inter-relationships among viruses were determined using a haemagglutination-inhibition (HI) assay with ferret antisera and visualised using antigenic cartography. All European isolates belonged to Florida Clade 2, all those from the USA belonged to Florida Clade 1. Two subpopulations of Clade 2 viruses were isolated, with either substitution A144V or I179V. Isolates from Dubai, obtained from horses shipped from Uruguay, belonged to Florida Clade 1 and were similar to viruses isolated in the USA the previous year. The neuraminidase (NA) sequence of representative strains from 2007 and 2009 to 2012 was also determined and compared with that of earlier isolates dating back to 1963. Multiple changes were observed at the amino acid level and clear distinctions could be made between viruses belonging to Florida Clade 1 and Clade 2.

Keywords: equine influenza virus, H3N8, surveillance, antigenic cartography, neuraminidase

Introduction

Equine influenza virus (EIV) is a major cause of respiratory disease in horses and spreads rapidly between naïve animals. Although rarely fatal in otherwise healthy horses, EIV can cause severe disruption to the racing and breeding industries. It can also cause more severe clinical signs in animals with concurrent disease, such as hyperadrenocorticism, or in those under physiological stress.

Influenza A viruses are subtyped according to their surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). HA mediates virus entry, by binding to sialic acid receptors on the host cell surface and mediating fusion of viral and host membranes (Skehel & Wiley, 2000). NA is involved in virus release from infected cells by cleaving sialic acid, it may also play a role in virus entry by allowing the virus to penetrate the mucus layer of the respiratory tract (Seto & Rott, 1966; Matrosovich et al, 2004).

Two subtypes of influenza are known to have infected horses, H3N8 and H7N7. Equine H7N7 was first isolated in 1956, equine H3N8 emerged in 1963 and spread globally over the following two years. Between 1963 and the late 1970s both subtypes co-circulated in horses and reassortment occurred between them, indicating the occurrence of mixed infections (Ito et al, 1999). There have been isolated reports of seroconversions to H7N7 in unvaccinated animals, however virus of this subtype has not been isolated since 1979 and has been considered extinct for 20 years (Madić et al., 1996; Webster, 1993). During the 1980s the H3N8 subtype diverged into 2 sub-lineages, Eurasian and American (Daly et al., 1996). The American lineage has since been divided further into the Kentucky, South American and Florida sublineages (Lai et al., 2001). More recently, the Florida sub-lineage has diverged into two clades, based on HA sequence and antigenic differences (Bryant et al., 2009; Lewis et al., 2011). Between 2006 and 2009, Florida clade 2 was seen predominantly in Europe with

occasional isolation of clade 1 strains in the UK and Ireland; in North America, recent isolates have all belonged to Clade 1 (Gagnon et al., 2007; Damiani et al., 2008; Bryant et al., 2011; Gildea et al., 2012).

Both clades have caused large outbreaks of equine influenza in the last 10 years. Examples include the UK in 2003 (Clade 2), Japan and Australia in 2007 (Clade 1), India in 2009 (Clade 2), Mongolia and China from 2008 to 2009 (Clade 2) and most recently in several countries in South America during 2012 (Clade 1) (Newton et al., 2006; Callinan, 2008; Ito et al., 2008; Yamanaka et al., 2008; Virmani et al., 2010; OIE-WAHID interface).

Vaccination is an effective method of control for equine influenza, providing protection by the induction of antibodies to viral surface glycoproteins, particularly HA. The role of antibodies to NA is unclear for EIV, however antibodies to human influenza NA are thought to contribute to protective immunity and neutralising titres correlate with reduced virus shedding in small animal models [Murphy et al., 1972; Brett & Johansson, 2005]. Like other influenza viruses, EIV undergoes antigenic drift and is able to evade antibody responses to divergent strains (Yates et al., 2000). Vaccine strains for equine influenza therefore need to be updated regularly and a formal process of vaccine strain selection is in place, overseen by the World Organisation for Animal Health (OIE). Genetic, antigenic and epidemiological data are considered prior to recommending changes to vaccine strains; current OIE recommendations are to include a representative of both Florida clade 1 and clade 2 viruses. To date, genetic and antigenic characterisation for selection of vaccine strains has focussed solely on the HA glycoprotein and antigenic drift in NA has been largely ignored.

Our aim was to improve the monitoring of field strains of EIV. Here we describe the establishment of a surveillance programme for EIV in the UK, to encourage the submission of equine nasal swab samples. We present the HA1 sequences and antigenic characterisation

of recent field strains from the UK, Dubai, Germany and USA and compare them with current OIE vaccine strain recommendations. We show that the Florida clade 1 and clade 2 viruses have diverged further since the OIE recommendation to include both in commercial vaccines. We also show that multiple changes have occurred within the NA gene segment of equine influenza H3N8 viruses since 1963.

Methods

Sentinel practice scheme

An invitation letter was sent to 60 veterinary practices with equine practitioners within the UK. Further practices were recruited to the scheme by invitation following submission of samples to the diagnostic laboratories at the Animal Health Trust (AHT). Participants were offered free diagnostic testing for samples from equids with suspected influenza, either nasal swabs or paired serum samples. A telephone helpline and dedicated website (www.equiflunet.org.uk) were also made available. Sampling packs were sent to each contributing veterinary practice, including submission forms, virus transport medium and swabs. Newsletters were also distributed to keep participants informed of relevant information. All positive diagnoses were followed up by telephone contact to collect epidemiological data, including vaccination histories of affected animals.

Diagnostic testing for presence of EIV

Nasopharyngeal swabs were taken from horses showing signs of acute respiratory disease, or close contacts of affected animals. Swabs were placed in sterile tubes containing 5 mL virus transport medium (PBS containing 200 U/mL streptomycin, 150 U/mL penicillin, 5µg/mL fungizone (Gibco) and 600 µg/mL tryptone phosphate broth). All equine nasopharyngeal swabs sent to the AHT from 2010 to 2011 were then assayed by an in house nucleoprotein-ELISA as described previously (Cook et al, 1988). Briefly, plates were coated with rabbit polyclonal serum against A/equine/Sussex/89 (H3N8), nasal swab extract was added to the plates and incubated for up to one hour. After washing, bound influenza antigen was detected by incubation with a monoclonal antibody to equine influenza virus nucleoprotein followed by anti-mouse peroxidase conjugated secondary antibody (Dako) and colorimetric assay. All

samples confirmed positive by NP ELISA were then subjected to RNA extraction and egg isolation as described below. Nasopharyngeal swabs sent to the AHT during 2012 were assayed by qRT-PCR as described previously (Bryant et al, 2010), using SensiFAST Probe Hi-ROX Onestep kit (Bioline) and a StepOne Plus qPCR instrument (Applied Biosystems). North American samples were tested by qRT-PCR (Lu et al, 2009) or by the Directigen™ Flu A test kit (BD, New Jersey, USA) according to the manufacturer's directions.

Diagnostic serology assay

The presence of antibodies to EIV in serum samples was determined by haemagglutination inhibition (HI) assay, using 1% chicken erythrocytes according to World Health Organisation standard procedures (WHO). For maximal sensitivity, sera were tested against Tween-treated viruses including A/equine/Prague/56 [H7N7], A/equine/Miami/63 [H3N8] and A/equine/Newmarket/2/93 [H3N8] (OIE 2012). Where possible, paired serum samples taken 14 days apart were analysed.

Virus isolation in eggs

Virus isolation was attempted from all swabs that were diagnosed positive by NP ELISA or qRT-PCR. Briefly, 0.1 ml each swab extract was inoculated into the allantoic cavity of two 10-day-old fertilized hen's eggs at neat, 10^{-1} and 10^{-2} dilutions and incubated at 34°C. Three days later, eggs were chilled at 4ºC overnight, allantoic fluid harvested and a haemaglutination (HA) assay performed to assess virus growth. Swabs giving a negative result after one round in eggs were passaged up to 3 times, checking for growth at each step to minimise the final number of passages. Working stocks were generated from virus isolates by inoculation of eggs at a 10^{-4} dilution, to reduce the risk of generating defective interfering particles. For most EIV strains, this was equivalent to approximately 10 to 100 EID_{50} per egg.

151 Antigenic characterisation by HI assay

152 Ferret antisera were raised against representative strains by intranasal instillation of 0.1 ml
153 EIV per nostril, equivalent to a final dose of $2x10^6$ EID_{50}. Sera were collected three weeks
154 post-infection and stored at -20°C. Prior to use, 300 µl each antiserum was incubated with
155 600 µl 0.38% potassium periodate for 15 min at room temperature, then 300 µl 3% glycerol-
156 PBS was added and the mixture incubated for a further 15 min at room temperature before
157 heat-inactivation at 56°C for 30 min. Equine antisera from AHT archives were raised by
158 aerosol challenge of Welsh mountain ponies and collected at least two weeks post-infection,
159 those against American strains were supplied by the Gluck Equine Research Center and
160 generated in the same manner from mixed-breed ponies. Equine sera were treated as
161 described for ferret. HI assays were carried out using a 96-well format, according to standard
162 procedures (WHO). Briefly, viruses were diluted to 4 HA units in a volume of 25 µl and
163 back-titrated to ensure accuracy. Two-fold serial dilutions of each ferret serum were prepared
164 in PBS and incubated with virus for 30-60 min at room temperature then 50 µl of 1% chicken
165 erythrocytes added. Samples were incubated at 4°C for 45 min prior to scoring. HI assays
166 were carried out at least twice and geometric means calculated. Isolates from different years
167 were grouped in separate batches, but each batch was run against the full panel of reference
168 antigens to allow comparison of data. Quantitative analyses of the ferret HI data were
169 performed using antigenic cartography, as described previously for human H3N2 and equine
170 H3N8 viruses (Smith et al., 2004; Lewis et al, 2011).

171 RNA extraction, RT PCR and sequencing

172 RNA was extracted from all ELISA-positive swabs using a QIAamp viral RNA mini kit
173 (Qiagen) according to the manufacturer's instructions. A 2-step PCR protocol was used,
174 comprising a reverse transcription (RT) step using uni-12 primer, 5'-AGCGAAAGCAGG-3'

and SuperScript II Reverse Transciptase from Invitrogen followed by PCR with either HA1-specific primers 5'-GCGAGCGAAAGCAGGGG-3' and 3'-GCGGATTTGCTTTTCTGGTAC-5' or NA-specific primers 5'-AGCAAAAGCAGGAGTTT-3' and 3'-AACTCCTTGTTTCTACT-5'. The PCR protocol consisted of an initial denaturation step of 92^{o}C for 5 minutes followed by 30 cycles of 95^{o}C for 1 minute, 50^{o}C for 1.5 minutes and 72^{o}C for 5.5 minutes. PCR products were separated by gel electrophoresis using a 1% agarose gel and visualised with GelRed (Biotium). PCR products were purified using kits supplied by Qiagen or Bioline, according to manufacturer's recommendations. PCR products were sequenced using ABI BigDye® Terminator v3.1 (Applied Biosystems) according to manufacturer's instructions on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Sequence analysis and Phylogenetic trees

Nucleotide sequences were visualized and edited using Seqman II version 5.03 (DNAstar Inc) and BioEdit (Ibis Pharmaceuticals Inc.). All sequences were deposited with Genbank. Nucleotide sequences were aligned to representative reference panels for HA1 or NA obtained from Genbank using ClustalW2 (EMBL-EBI). Derived amino acid sequences were aligned against representative strains from each sublineage of EIV, including pre-divergence, Eurasian, American (Kentucky), Florida Clades 1 and 2. Maximum-likelihood (ML) phylogenetic trees for the nucleotide sequences encoding HA1 (1009 nt) and NA (1410 nt) were created using PhyML version 3 (Guindon et al., 2009). Amino acid alignments were generated separately for isolates compared against A/equine/Richmond/1/07 using BioEdit version 7.0.5.3 (Ibis Pharmaceuticals Inc.).

Results

Outbreaks and sample submission: 2010 to 2012

Equine influenza outbreaks within the UK that were diagnosed from either nasal swabs or paired serum samples between January 2010 and December 2012 are summarised in Table 1. Twenty one counties were affected in England, Wales and Scotland with multiple outbreaks in some areas. In addition to samples submitted from the UK, 17 virus isolates obtained between 2010 and 2012 were received from the Gluck Equine Research Center, OIE reference laboratory for equine influenza in the USA (supplementary data). Outbreaks in the USA were reported from 6 states in 2010, 10 states in 2011 and 17 in 2012, many of which were described by Pusterla et al. (2011) and subsequent work by that group. During 2011 and 2012, there were also outbreaks of equine influenza reported in Germany, including cases in vaccinated animals. One isolate from 2012 was submitted to the AHT by the OIE reference laboratory for Germany, for antigenic characterisation. Three virus isolates were also submitted from the Central Veterinary Laboratories, Dubai, following an outbreak in a quarantine facility. The source of infection was a group of endurance horses transported from Uruguay to Dubai, consistent with reports to the OIE of extensive outbreaks of equine influenza affecting around 2,500 horses in Uruguay during 2012. The endurance horses had received a primary course of two doses of vaccine, according to the manufacturer's recommendations.

Genetic analyses - HA

HA1 sequences were obtained from all virus isolates, plus one swab sample from which virus could not be isolated (East Renfrewshire/1/11). For most isolates, sequence was also determined directly from the nasal swab extract. In all instances, the HA1 nucleotide

sequence obtained from both egg isolate and swab extract was identical, suggesting that no significant selection had occurred during egg passage. Phylogenetic analysis was carried out for the recent isolates against a panel of 130 equine H3N8 HA1 nucleotide sequences from GenBank. The resulting analysis grouped the viruses into 5 well-defined clusters, corresponding to the Pre-divergent, Eurasian, American and Florida clade 1 and 2 sublineages (Figure 1). These clusters were each supported by high bootstrap values, ranging from 97 to 100 after 100 replicates. All viruses isolated in the UK between 2010 and 2012 belonged to the Florida clade 2 sublineage, all those characterised from the USA were of the Florida clade 1 sublineage. The strains from Dubai were most similar to clade 1 isolates from Kentucky 2011. Derived amino acid sequences were aligned against the current OIE recommended strain for Florida clade 2 (A/equine/Richmond/1/07), shown in Figure 2. In the alignment, each strain is representative of multiple isolates with identical HA1 sequences.

The Florida clade 2 viruses appeared to have 3 consistent amino acid substitutions compared to Richmond/1/07, two of which were P103L and V112I (Figure 2). A further substitution, E291D, was observed between Richmond/1/07 and all of the other strains described here; this was shared by all the clade 2 viruses isolated in the UK during 2007 (Bryant et al, 2009). Two different sub-populations were isolated in the UK during the period studied here, those with an additional change at position 144 and those with a substitution at 179, which was also observed in recent isolates from France and Germany. Amongst the American clade 1 viruses, three amino acid substitutions were unique to 2010 isolates from California: D31N, T163I and I230V. There were 5 consistent amino acid changes between the current clade 1 OIE-recommended strain (South Africa/4/03) and isolates from 2009 onwards: G7D, R62K, D104N, A138S and V223I. The HA2 sequence for some of the isolates was also determined which revealed amino acid substitutions between the recommended vaccine strains and the most recent Florida clade 1 and 2 viruses (data not shown). These substitutions in HA2

included N169S and L187M between Richmond/1/07 and clade 2 isolates, and I198V between South Africa/4/03 and the clade 1 isolates. There were five amino acid substitutions in HA2 between the most recent clade 2 and clade 1 isolates (T43A, E50G, N169S, L187M and I198V).

The conserved amino acid substitutions between the Florida clade 1 and Florida clade 2 viruses were mapped to the structure of the trimeric HA molecule, using A/duck/Ukraine/68 [H3N8] PDB 1MQL (Ha et al, 2003), shown in Figure 3. Four differences mapped to the top of the molecule, close to the receptor binding site, with a further 12 mapping on the surface of the molecule. For comparison, the differences between the clade 2 viruses and the OIE recommended strain Richmond/1/07 and the clade 1 viruses and the recommended strain South Africa/4/03 are also shown. The clade 1 versus clade 2 comparison shows multiple differences between strains, whereas the OIE-recommended strain for each clade shows only one or two differences on the top of the HA molecule.

Genetic analyses – NA

To investigate the level of variation in NA amongst circulating strains, the nucleotide sequence of segment 6 was determined for 19 strains isolated between 2010 and 2012 from the UK, USA, Germany and Dubai, as well as representative clade 1 and 2 isolates from 2007 and 2009. Phylogenetic analyses were carried out as described for HA, using a panel of 99 sequences from Genbank from 1963 to 2011 (Figure 4). The topology of the NA tree was similar to that of HA and separation of viruses belonging to Florida clades 1 and 2 was well-supported with a bootstrap value of 98%. Major clades also correlated with significant country-wide outbreaks in 1979, 1989, 2003 and 2007. Three recent Florida clade 1 isolates, (Dorset/09, Lanarkshire/09 and Yorkshire/3/09) had an NA segment that was more similar to those of the clade 2 viruses and one Florida clade 2 isolate (Perthshire/1/09) had an NA more

similar to those of clade 1 viruses, indicating that reassortment had taken place between clade 1 and clade 2. These isolates are highlighted in Figure 4.

Derived amino acid sequences were aligned against representative strains from four major clades identified by phylogenetic analysis and are shown in Figure 5. Multiple amino acid substitutions were observed between sublineages with signature substitutions readily identified for the current Florida clade 1 and clade 2 viruses compared with the older American strains. There were 16 amino acid substitutions between the most recent UK isolates from clade 1 and clade 2. The majority of changes occurred within the first 80 residues, including the membrane anchor sequence and stalk region of NA; these are not included in the protein structure solved for various subtypes of NA. For the purpose of structure mapping, the amino acid numbering of the predicted ectodomain was adjusted to correspond to that present in the H5N1 and H3N8 NA protein structure database files 2HTY and 2HT5. Multiple changes occurred on the surface of NA, shown mapped on the tetrameric structure of H5N1 (Figure 6), affecting both the distal and proximal surfaces of the molecule. An additional substitution V147I within the 150 loop was observed in equine viruses from the Japanese and Australian outbreak in 2007.

Antigenic characterisation

Low passage virus isolates were characterised by HI assay using post infection ferret antisera raised against eight representative EIV strains and the homologous reference strains. Sera included those raised against representatives from relevant sublineages [American (Kentucky), Florida clade 1, Florida clade 2], current UK vaccine strains and OIE recommended strains, as indicated in Table 2. All virus isolates raised low titres against the

European sera (data not shown), as expected from their genetic characterisation. Ferret antisera against the American Kentucky lineage vaccine strains Newmarket/1/93 and Kentucky/98 recognised the Florida clade 2 isolates from 2010 to 2012, but gave a slightly lower titre than against homologous strains. They typically showed a 2-fold difference for Kentucky/98 and 2- to 4-fold lower titre for Newmarket/1/93. These sera recognised the Florida clade 1 viruses poorly, with most strains showing a 16- to 64- fold reduction against Newmarket/1/93 and an 8- to 16-fold lower titre against Kentucky/98 compared to homologous antigen. Ferret antisera were raised against the early representative of the Florida clade 2 viruses, Kentucky/97, which has sequence similarity to the older American strains, and outbreak strains Newmarket/5/03 and Richmond/1/07 (the current OIE-recommended clade 2 strain). These sera all recognised the clade 2 isolates from 2010 to 2012 and in most instances, to at least the same level as their respective homologous strains. None of the sera raised the maximum titre against their homologous strains; this was particularly noticeable for Kentucky/97 (Table 2). Most of the clade 2 field isolates gave a 2- to 4-fold higher titre than Kentucky/97. All three of the antisera raised to clade 2 viruses gave lower titres against the clade 1 isolates from 2010 to 2012 than the clade 2 strains, reflecting the genetic differences between the two groups. Three sera were raised against clade 1 strains, including South Africa/4/03, one of the current OIE recommended strains for vaccines. The reciprocal pattern was seen with these sera, all sera recognised the clade 1 field isolates with high titres but with lower titres against the clade 2 strains. In both directions, 8- to 16-fold differences in titre were common suggesting antigenic divergence between the two clades.

Antigenic cartography

The antigenic relationships between 44 equine influenza A(H3N8) viruses, including the recent isolates described above and a reference panel of representative Florida clade 1 and

clade 2 strains are shown in Figure 7. The viruses grouped into two distinct antigenic clusters, with all the Florida clade 2 isolates falling into the blue cluster and all the clade 1 isolates grouping together in the red cluster. This is consistent with our previous findings, showing that the two phylogenetic clades were antigenically distinct (Lewis et al, 2011). The current recommended vaccine strains, A/equine/Richmond/1/07 and A/equine/South Africa/4/03, for either clade were located within their respective clusters and the antigenic distance between each isolate and the representative strain did not exceed 2.1 antigenic units, equivalent to a 4-fold difference in HI titre. When we measured the antigenic distances from ferret sera raised to current and previous OIE-recommended strains for the Clade 2 cluster, we found that on average, currently circulating strains were 1.5 antigenic units from the A/equine/Richmond/1/2007 serum, but on average 2 antigenic units from a previously recommended vaccine strain, A/equine/Newmarket/1/1993 serum.

HI of strains against Richmond and South Africa equine sera

To determine whether the antigenic differences between strains belonging to the American, Florida clade 1 and Florida clade 2 sublineages could be distinguished by equine sera, HI assays were carried out against a panel of post-infection equine sera for a representative selection of strains from the three groups (Table 3). Compared with ferret sera, the titres were lower for equine sera, with homologous titres only reaching 128 rather than up to 1024. The two American sera showed lower titres against viruses from both of the Florida sublineages than the American strains; the antiserum raised against the older strain Kentucky/91 raised even lower titres than Kentucky/99. Although differences were subtle, these sera appeared to recognise the clade 1 strains better than clade 2, in contrast to ferret sera raised against similar strains (Table 2).

Of the sera raised against more recent viruses, both sera from Richmond/1/07-infected ponies recognised all three groups of viruses, with no more than a 2-fold difference in titre between field isolates and Richmond/1/07. The results for the South Africa clade 1 sera were slightly different, with the highest titres raised against homologous or very closely related clade 1 viruses. Differences in HI titre within this group of viruses were less than 2-fold compared with the homologous titre for South Africa/4/03. However, the clade 1 sera consistently raised lower HI titres against both American and clade 2 viruses, giving differences of 2- to 4-fold compared with the South Africa/4/03 strain.

Discussion

Equine influenza viruses belonging to the H3N8 subtype are thought to have crossed the species barrier from birds in the early 1960s and subsequently spread worldwide. They have continued to circulate since then, causing widespread outbreaks in naïve populations, such as South Africa in 2003 and Australia in 2007, but also occasionally in vaccinated horses. Extensive outbreaks in Europe in highly vaccinated horses in the late 1980s demonstrated that circulating equine influenza viruses had undergone significant antigenic drift from the strains included in vaccines and highlighted the need for effective surveillance programmes.

In 2003, two large scale epidemics of equine influenza in the UK and South Africa led to the division of the Florida sublineage of equine influenza into two clades, 1 and 2. The original difference was based upon two amino acid substitutions within HA1 at positions 78 and 159, which were sufficient to cause an antigenic difference that was recognisable by ferret sera (OIE, 2008). Since then, the two clades have diverged further and we show here that there are now 9 consistent amino acid differences between viruses from the different groups isolated in 2012 with a further 2 substitutions between subsets of the clade 2 viruses. Additional variants arose during this period as a result of reassortment between clade 1 and clade 2 viruses, which led to new combinations of HA and NA.

In 2010, the OIE recommended that vaccine manufacturers should include a representative of both Florida clade 1 and Florida clade 2 viruses (OIE 2010). Surveillance data reported here confirm that viruses from both clades continued to circulate, with viruses of Florida clade 1 likely to be the cause of multiple large-scale outbreaks in South America. Clade 2 viruses predominated in Europe, causing outbreaks in the UK, France, Ireland and Germany. Surveillance data therefore support inclusion of both clades in vaccines for horses that travel between continents.

376 Antigenic characterisation of the strains reported here indicated that representatives of the
377 Florida clades 1 and 2 could be distinguished from each other readily, with differences
378 ranging between 2- and 16-fold by HI. These results were comparable to those seen with
379 viruses isolated in 2009 (Bryant et al., 2011), suggesting that the ferret model does not
380 distinguish between viruses with HA1 molecules containing the most recent amino acid
381 differences. These substitutions were primarily conservative changes, the notable exception
382 being the P103L substitution seen in recent clade 2 viruses. However, this position is buried
383 within the HA molecule and is not likely to affect the binding of ferret antisera. Similarly,
384 ferret sera raised against Kentucky lineage vaccine strains from the 1990s continued to
385 recognise Florida clade 2 viruses. However, there was typically a difference of 4-fold
386 between the titres against the most recent clade 2 viruses from 2011 and 2012 compared with
387 the homologous American reference strains Newmarket/1/93 and Kentucky/98, whereas
388 some of those from 2010 showed only a 2-fold difference. Caution should be observed in
389 interpretation of cross-reactive titres, as ferret sera to Newmarket/1/93 vaccine strain cross-
390 reacted well against the Newmarket/5/03 outbreak strain from the 2003 epidemic in the UK,
391 however commercial vaccines containing this strain failed to protect against the outbreak
392 strain (Newton et al., 2006). Cross-reaction was therefore a poor indicator of cross protection
393 in this instance. Clade 1 ferret sera cross-reacted well against other members of the group and
394 also recognised clade 2 viruses, but to a lower level. Interestingly, equine sera raised against
395 Richmond/1/07 reacted well against viruses belonging to all 3 sublineages whereas South
396 Africa/4/03 did not react as well against the clade 2 viruses. This result suggests that
397 Richmond/1/07 could make a good vaccine strain for protecting against both clade 1 and
398 clade 2 viruses, with the caveat outlined above that cross-reaction does not necessarily
399 correlate with cross-protection. Ideally, equine sera would be used for antigenic
400 characterisation of equine influenza virus strains, however these sera are often broadly cross-

401 reactive making them insensitive to antigenic differences between strains. In our experience,
402 the HI titres are often low and strains that give a 4- or 8-fold difference with ferret sera may
403 only raise a 2-fold difference using equine sera, making interpretation difficult.

404 To date, vaccine strain selection for EIV has focussed solely on HA genetic and antigenic
405 differences and there is a lack of sequence data available for NA for recent isolates from
406 Florida clade 2. Comparison of amino acid sequences shown here indicated that NA had
407 undergone considerable divergence since 1963 and outbreak strains from 1979, 1989, 2003
408 and 2007 had multiple amino acid changes compared with previous sublineages. Recent
409 Florida clade 1 and clade 2 viruses differed by 16 or more amino acids and it was clear that
410 reassortment had taken place in strains isolated in the UK. Multiple changes occurred within
411 the stalk region of NA and so could not be mapped on the structure of the tetrameric head
412 region of the NA; however changes also occurred on both the upper and lower surfaces of the
413 head region, including regions close to the active site and within the C-terminal region where
414 human sera have been shown to recognise epitopes in the NA of H5N1 viruses (Khurana et
415 al., 2011). Also of interest was the V147I substitution observed in isolates from the 2007
416 outbreaks in Japan and Australia. All other H3N8 viruses of avian or equine origin, with NA
417 sequences currently available on Genbank, had valine at this position. The exceptions were
418 the H3N8 canine isolates from the USA, which had isoleucine (data not shown). This
419 position is equivalent to V149 of the N1 structure and falls within the 150 loop of NA
420 (Russell et al, 2006), an important region for NA activity (Lin et al. 2010). The conformation
421 of this loop may affect the size of the adjacent 150 cavity, which potentially differs between
422 group 1 and group 2 NAs.

423 Further work is required to determine the role of antibodies to NA in immunity in the horse
424 but they may contribute to vaccine efficiency and immune selection (Johansson et al., 1998).

Conclusion

Equine influenza Florida sublineage clade 1 and 2 viruses continued to cause outbreaks worldwide between 2010 and 2012. Clade 2 predominated in Europe while clade 1 was isolated in North and South America. Sequence analysis of NA revealed that reassortment had occurred between the two clades and some virus isolates from 2009 had new combinations of HA and NA. The two sublineages have diverged further since 2009 and can be distinguished readily by antigenic analysis. Current OIE vaccine strain recommendations for representatives of Florida clade 1 and clade 2 remain adequate, based on antigenic differences determined by HI.

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449 (University of Kentucky).

450

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571

572 **Tables**

573 Table 1. Outbreaks of EIV in the UK 2010 to 2012.

574 Footnote: NP ELISA – nucleoprotein enzyme linked immunosorbent assay, qPCR –
575 quantitative polymerase chain reaction, HI – haemagglutination inhibition assay, HA Acc. –
576 Haemagglutinin accession numbers, NA Acc. – Neuraminidase accession numbers.

577

578 Table 2. HI titres of EIV strains using ferret sera.

579 Footnote: The lineage of new isolates is indicated on the left and ordered by isolation
580 date. Homologous titres are shown in bold, titres for sera against strains from the same
581 sublineage are highlighted in grey boxes. New/1/93 – A/eq/Newmarket/1/93, Ken/97 –
582 A/eq/Kentucky/97, Ken/98 – A/eq/Kentucky/98, New/5/03 – A/eq/Newmarket/5/03,
583 SA/4/03 – A/eq/South Africa/4/03, Rich/1/07 – A/eq/Richmond/1/07, Lin/1/07 –
584 A/eq/Lincolnshire/1/07, Dor/09 – A/eq/Dorset/09. Am – American lineage, FC1 – Florida
585 sublineage clade 1, FC2 - Florida sublineage clade 2.

586

587 Table 3. HI titres of EIV strains using post-infection equine sera.

588 Footnote: The lineage of strains tested is indicated on the left. Titres for sera against
589 strains from the same sublineage are highlighted in grey boxes, homologous titres are shown
590 in bold. Ken/91 – A/eq/Kentucky/91, Ken/99 - A/eq/Kentucky/99, Rich/07 -
591 A/eq/Richmond/1/07, Ken/4/07 – A/eq/Kentucky/4/07, SA/4/03 – A/eq/South Africa/4/03.
592 For Rich/07 and SA/4/03 antisera, numbers 1 and 2 relate to serum samples from pony 1 and

593 pony 2 respectively. Am – American lineage, FC1 – Florida sublineage clade 1, FC2 -
594 Florida sublineage clade 2.

595

596 Supplementary data

597 Table S1 Virus isolates from outside the UK: Genbank accession numbers. FC1 – Florida
598 sublineage clade 1, FC2 - Florida sublineage clade 2.

599

600 **Figure captions**

601 **Figure 1**

602 HA Phylogenetic Tree

603 Phylogenetic analysis of HA1 nucleotide sequences encoded by EIV. A maximum likelihood
604 tree was created using PhyML version 3. Bootstrap values obtained after 100 replicates are
605 shown at major nodes. Amino acid substitutions are indicated at branch points, or in brackets.
606 Phylogenetic groups are shown by continuous bars on the right, as indicated. Accession
607 numbers for the sequences reported in this manuscript are listed in Table 1. Sequences are
608 coloured by date of isolation for the years 2010 (green), 2011 (red) and 2012 (blue) with the
609 older isolates in black. Representative OIE-recommended vaccine strains
610 A/eq/Richmond/1/07 and A/eq/South Africa/4/03 are shown in bold. Reassortant strains
611 containing HA from one Florida clade and NA from the other, identified in this manuscript,
612 are highlighted in yellow.

613 **Figure 2**

614 Alignment of HA1 amino acid sequences.

615 Derived amino acid differences in HA1 observed between representative field strains from
616 Florida clade 2 and clade 1 isolated during 2010-2012 compared to A/eq/Richmond/1/07
617 (top). The sublineage of isolates is indicated on the left and ordered by isolation date.
618 Residues are numbered from 1 to 329 starting with the serine residue downstream of the
619 predicted signal peptide. Amino acid identity to A/eq/Richmond/1/07 is shown with a dot.
620 Examples of strains from 2009 are included to allow comparison with Bryant et al (2011).
621

622 **Figure 3**

623 HA structures.

624 Location of the amino acid differences on the A/duck/Ukraine/1/63 H3 HA structure (Ha et
625 al, 2003) between OIE recommended Florida clade 1 strain A/eq/South Africa/4/03 and
626 2010-2012 clade 1 isolates, OIE recommended Florida clade 2 strain A/eq/Richmond/1/07
627 and 2010-2012 clade 2 isolates and 2010-2012 isolates from Florida clade 1 and clade 2.
628 Established changes are shown in red, those that appeared in one year only are shown in blue.
629 HA1 residues 112, 179, 230 and 251 are buried. HA1 residue 7 and HA2 residues 187 and
630 198 are not shown.

631 **Figure 4**

632 NA Phylogenetic Tree

633 Phylogenetic analysis of NA nucleotide sequences encoded by EIV. A maximum likelihood
634 tree was created using PhyML version 3. Bootstrap values obtained after 100 replicates are
635 shown at major nodes. Amino acid substitutions are indicated at branch points, or in brackets.
636 Phylogenetic groups are shown by continuous bars on the right, as indicated. Accession
637 numbers for the sequences reported in this manuscript are listed in Table 1. Sequences are
638 coloured by date of isolation for the years 2010 (green), 2011 (red) and 2012 (blue) with the
639 older isolates in black. Representative OIE-recommended vaccine strains
640 A/eq/Richmond/1/07 and A/eq/Ohio/03 (A/eq/South Africa/4/03-like) are shown in bold.
641 Reassortant strains containing NA from one Florida clade and HA from the other, identified
642 in this manuscript, are highlighted in yellow.

643 **Figure 5**

644 Alignment of NA amino acid sequences.

Derived amino acid differences in NA observed between representative strains from Pre-divergent, American, Eurasian, Florida clade 1 and Florida clade 2 sublineages compared to A/eq/Miami/63 (top). The sublineage of isolates is indicated on the left and ordered by isolation date. Residues are numbered from 1 to 470 starting with the methionine residue at the start of the predicted signal peptide. The numbering has not been adjusted to correspond to N1 or N2 numbering. Amino acid identity to A/eq/Miami/63 is shown with a dot.

Figure 6

NA structure

Location of the amino acid differences on the H5N1 NA structure (Russell et al., 2006) between OIE recommended Florida clade 2 strain A/eq/Richmond/1/07 and 2010-2012 clade 2 isolates, OIE recommended Florida clade 1 strain A/eq/South Africa/4/03 and 2010-2012 clade 1 isolates, as well as 2010-2012 isolates from Florida clade 1 and clade 2. Established changes are shown in red, those that appeared in one year only are shown in blue.

Figure 7

Antigenic cartography

Antigenic map showing the relationships between virus strains isolated between 2010 and 2012. Virus strains are shown as spheres, the positions of sera are shown as open boxes. The OIE-recommended vaccine strains are indicated in black (A/eq/South Africa/4/2003 & A/eq/Richmond/1/2007) and their corresponding sera as larger squares; representative strains from clade 1 (A/eq/Lincolnshire/1/2007, A/eq/Dorset/2009), clade 2 (A/eq/Kentucky/1997, A/eq/Newmarket/5/2003) and current commercial vaccine strains(A/eq/Newmarket/1/1993,

A/eq/Kentucky/1998) are highlighted in turquoise. The scale bar indicates one antigenic unit, equivalent to a 2-fold difference in HI titre.

Table 1

Date	Location	Detection method	Premises	VI	Virus Name	HA acc.	NA acc.
May-10	Lincolnshire	NP ELISA	Sanctuary, 150+ affected	5	A/eq/Lincolnshire/1/10	KF026381	-
May-10	Shropshire	NP ELISA	Private yard, all of 4 affected	1	A/eq/Shropshire/10	KF026378	KF049195
Jun-10	Surrey	NP ELISA	Riding school, 10 affected out of 30	3	A/eq/Surrey/2/10	KF026376	-
					A/eq/Surrey/4/10	KF026377	-
Jul-10	Nottinghamshire	NP ELISA	Small private yard, 1 affected	1	A/eq/Nottinghamshire/1/10	KF026379	-
Aug-10	Worcestershire	NP ELISA	Private yard	1	A/eq/Worcestershire/1/10	KF026375	-
Aug-10	Nottinghamshire	HI	Private yard, 5 affected	-	-	-	-
Aug-10	Lanarkshire	NP ELISA	Livery/riding stables, 1 affected and isolated, 30 unaffected	1	A/eq/Lanarkshire/1/10	KF026380	-
							-
Sep-10	Hampshire	NP ELISA	Unknown, 2 out of 2 ponies affected	2	A/eq/Hampshire/1/10	KF026382	-
					A/eq/Hampshire/2/10	KF026383	-
Sep-10	Cumbria	NP ELISA	Private yard, 1 horse affected for 10 days before sampling, other horses unaffected	-	-	-	-
Nov-10	Leicestershire	NP ELISA	Unknown, weak positive	0	-	-	-
Jun-11	Cardiff	NP ELISA	1 affected	0	-	-	-
Aug-11	Wiltshire	HI	1 affected	-	-	-	-
Aug-11	Lanarkshire	NP ELISA	Private yard, 1 affected	1	A/eq/Lanarkshire/1/11	KF026385	-
Aug-11	Surrey	NP ELISA	Livery yard, 2 affected	1	A/eq/Surrey/1/11	KF026384	-
Sep-11	Lanarkshire	NP ELISA	Private yard, 1 affected	1	A/eq/Lanarkshire/2/11	KF026386	-
Oct-11	Kent	NP ELISA	Eventing yard, 2 affected	1	A/eq/Kent/1/11	KF026387	-
Oct-11	Devon	NP ELISA	Sanctuary, 2+ affected	2	A/eq/Devon/1/11	KF026389	KF049194
					A/eq/Devon/2/11	KF026390	-
Nov-11	Berkshire	HI	Hunt yard, 2 affected	-	-	-	-
Nov-11	East Renfrewshire	NP ELISA	Livery yard, 3 affected	1	A/eq/East Renfrewshire/2/11	KF026388	KF049172
				-	A/eq/East Renfrewshire/1/11	KF049198	-
Dec-11	Cheshire	NP ELISA	Training yard, 3 affected	1	A/eq/Cheshire/1/11	KF026391	-
Apr-12	Lancashire	qPCR	Training yard, weak positive, 3 affected	0	-	-	-
Sep-12	Essex	qPCR	Private yard, weak positive, 3 affected	0	-	-	-
Oct-12	County Durham	qPCR	Livery yard, weak positive, 3 affected	0	-	-	-
Nov-12	Roxburghshire	qPCR	3 affected	1	A/eq/Roxburghshire/1/12	KF026395	KF049190
Nov-12	County Durham	qPCR	Private yard, 6 affected	2	A/eq/County Durham/2/12	KF026396	KF049192
Nov-12	Worcestershire	qPCR	Riding centre, 10 affected	2	A/eq/Worcestershire/1/12	KF026375	KF049174
					A/eq/Worcestershire/2/12	KF026392	KF049188
Nov-12	Herefordshire	qPCR	Private yard, 2 affected	0	-	-	-
Nov-12	Wiltshire	HI	Riding school, 12 affected	-	-	-	-
Nov-12	Worcestershire	qPCR	Livery yard, 2 affected	2	A/eq/Worcestershire/3/12	KF026393	-
					A/eq/Worcestershire/4/12	KF026394	KF049189

Table 2

	REFERENCE FERRET ANTISERA							
	New/1/93 Am	Ken/98 Am	Ken/97 FC2	New/5/03 FC2	Rich/1/07 FC2	SA/4/03 FC1	Lin/1/07 FC1	Dor/09 FC1
REFERENCE STRAINS								
Newmarket/1/93	**512**	256	512	128	512	32	45	64
Kentucky/98	256	**256**	512	362	256	32	11	64
Kentucky/97	64	32	**128**	128	512	128	91	128
Newmarket/5/03	128	128	256	**256**	512	181	256	64
Richmond/1/07	128	128	256	256	**512**	64	181	64
South Africa/4/03	32	32	128	128	128	**512**	1024	512
Lincolnshire/07	64	64	128	128	128	1024	**1024**	724
Dorset/09	64	64	128	128	256	512	1024	**1024**
FLORIDA CLADE 2								
Perthshire/1/09	128	64	128	128	512	64	64	64
Yorkshire/09	128	91	128	128	512	128	32	64
Lincolnshire/1/10	181	181	512	362	724	181	91	181
Shropshire/10	256	128	512	256	512	128	64	128
Surrey/2/10	256	256	1024	512	512	128	64	128
Lanarkshire/10	128	128	181	181	256	128	32	64
Hampshire/2/10	128	128	362	181	512	91	64	128
Lanarkshire/1/11	128	128	512	512	512	128	128	128
Surrey/1/11	181	128	512	724	512	128	91	128
Lanarkshire/2/11	128	128	256	256	362	64	64	64
Kent/1/11	128	128	512	256	512	128	64	64
Devon/1/11	91	64	256	181	256	64	45	64
East Renfrewshire/2/11	128	128	362	256	512	128	128	128
Cheshire/1/11	128	128	256	256	256	128	91	128
Roxburghshire/12	256	256	2048	512	1448	256	256	256
Co. Durham/2/12	128	128	256	256	512	64	64	64
Co. Durham/3/12	128	128	512	256	512	128	128	128
Worcestershire/1/12	128	128	362	256	512	181	181	91
Worcestershire/2/12	256	256	1024	1024	1024	256	256	256
Worcestershire/3/12	181	256	512	362	1024	128	128	128
Worcestershire/4/12	181	256	1024	512	1024	256	181	181
Lichtenfeld/1/12	64	128	512	256	362	128	256	128
FLORIDA CLADE 1								
California/2/10	8	11	45	45	45	512	512	512
Oregon/1/10	16	23	91	91	91	724	724	724
Kentucky/1/11	32	64	128	128	362	1024	1024	1024
New York/1/11	11	32	64	64	64	512	362	512
Pennsylvania/2/11	<8	8	32	32	32	128	128	128
Pennsylvania/3/11	<8	16	64	64	64	512	512	512
Pennsylvania/5/11	<8	11	45	32	64	256	256	256
Pennsylvania/6/11	16	16	128	64	128	512	724	1024
Dubai/1/12	16	64	256	128	181	1024	1024	1448
Kentucky/1/12	8	32	128	64	128	1024	724	724
Kentucky/2/12	8	32	128	128	91	512	512	724
Kentucky/3/12	8	32	64	64	128	512	512	512
Kentucky/5/12	<8	16	64	64	64	256	362	362
Texas/1/12	16	64	128	128	128	724	1024	1024

Table 3

	REFERENCE EQUINE ANTISERA						
	Ken/91 Am	Ken/99 Am	Rich/07 1 FC2	Rich/07 2 FC2	Ken/4/07 FC1	SA/4/03 1 FC1	SA/4/03 2 FC1
REFERENCE STRAINS							
AMERICAN							
Newmarket/1/93	64	64	256	256	91	64	64
Kentucky/98	128	128	256	256	128	64	45
FLORIDA CLADE 2							
Kentucky/97	16	64	128	128	91	64	64
Newmarket/5/03	45	91	181	181	91	45	64
Richmond/1/07	<8	32	**128**	**128**	45	32	32
Shropshire/10	32	91	256	256	128	64	64
FLORIDA CLADE 1							
South Africa/4/03	45	91	128	181	128	**128**	**128**
Lincolnshire/1/07	91	91	256	256	128	91	128
Dorset/09	45	91	256	256	128	91	181

Figure 1

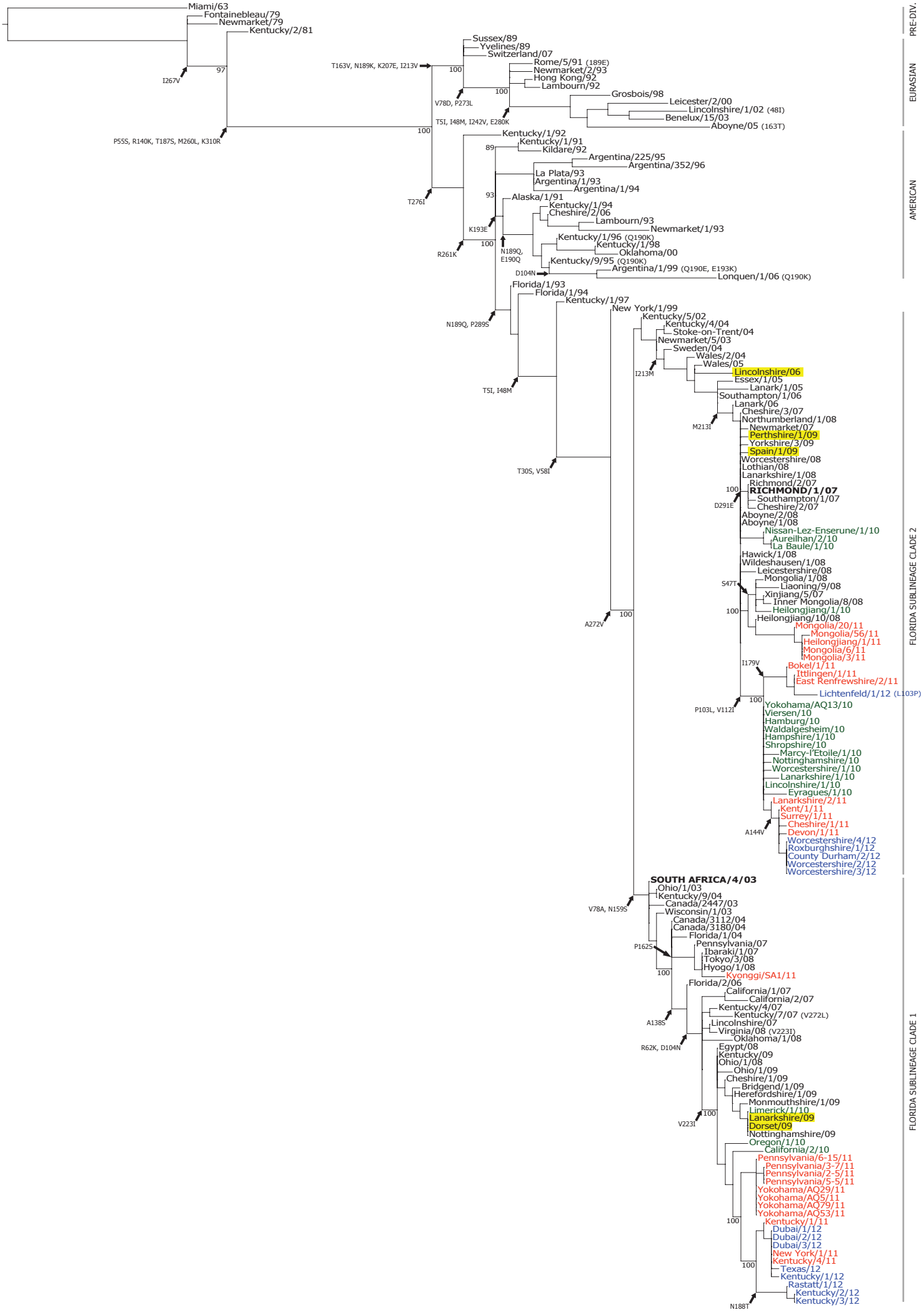

Miami/63
Fontainebleau/79
Newmarket/79
Kentucky/2/81
I267V
97
P55S, R140K, T187S, M260L, K310R
Sussex/89
Yvelines/89
Switzerland/07
T163V, N189K, K207E, I213V
100
Rome/5/91 (189E)
Newmarket/2/93
Hong Kong/92
Lambourn/92
100
V78D, P273L
Grosbois/98
Leicester/2/00
Lincolnshire/1/02 (48I)
Benelux/15/03
Aboyne/05 (163T)
T5I, I48M, I242V, E280K
100
Kentucky/1/92
89
Kentucky/1/91
Kildare/92
Argentina/225/95
Argentina/352/96
La Plata/93
Argentina/1/93
Argentina/1/94
T276I
93
Alaska/1/91
Kentucky/1/94
Cheshire/2/06
Lambourn/93
Newmarket/1/93
K193E
Kentucky/1/96 (Q190K)
Kentucky/1/98
Oklahoma/00
100
R261K
N189Q, E190Q
Kentucky/9/95 (Q190K)
Argentina/1/99 (Q190E, E193K)
D104N
Lonquen/1/06 (Q190K)
Florida/1/93
Florida/1/94
Kentucky/1/97
N189Q, P289S
New York/1/99
Kentucky/5/02
Kentucky/4/04
Stoke-on-Trent/04
Newmarket/5/03
Sweden/04
Wales/2/04
Wales/05
I213M
Lincolnshire/06
Essex/1/05
Lanark/1/05
Southampton/1/06
Lanark/06
Cheshire/3/07
Northumberland/1/08
M213I
Newmarket/07
Perthshire/1/09
Yorkshire/3/09
Spain/1/09
Worcestershire/08
Lothian/08
Lanarkshire/1/08
Richmond/2/07
T5I, I48M
T30S, V58I
100
RICHMOND/1/07
D291E
Southampton/1/07
Cheshire/2/07
Aboyne/2/08
Aboyne/1/08
Nissan-Lez-Enserune/1/10
Aureilhan/2/10
La Baule/1/10
Hawick/1/08
Wildeshausen/1/08
Leicestershire/08
Mongolia/1/08
S47T
Liaoning/9/08
Xinjiang/5/07
Inner Mongolia/8/08
100
Heilongjiang/1/10
Heilongjiang/10/08
Mongolia/20/11
Mongolia/56/11
Heilongjiang/1/11
Mongolia/6/11
Mongolia/3/11
A272V
100
I179V
Bokel/1/11
Ittlingen/1/11
East Renfrewshire/2/11
Lichtenfeld/1/12 (L103P)
100
P103L, V112I
Yokohama/AQ13/10
Viersen/10
Hamburg/10
Waldalgesheim/10
Hampshire/1/10
Shropshire/10
Marcy-l'Etoile/1/10
Nottinghamshire/10
Worcestershire/1/10
Lanarkshire/1/10
Lincolnshire/1/10
Eyrague/1/10
Lanarkshire/2/11
Kent/1/11
Surrey/1/11
Cheshire/1/11
Devon/1/11
A144V
Worcestershire/4/12
Roxburghshire/1/12
County Durham/2/12
Worcestershire/2/12
Worcestershire/3/12
SOUTH AFRICA/4/03
Ohio/1/03
Kentucky/9/04
V78A, N159S
Canada/2447/03
Wisconsin/1/03
Canada/3112/04
Canada/3180/04
Florida/1/04
P162S
Pennsylvania/07
Ibaraki/1/07
Tokyo/3/08
Hyogo/1/08
100
Kyonggi/SA1/11
Florida/2/06
California/1/07
California/2/07
Kentucky/4/07
A138S
Kentucky/7/07 (V272L)
Lincolnshire/07
Virginia/08 (V223I)
Oklahoma/1/08
R62K, D104N
Egypt/08
Kentucky/09
Ohio/1/08
Ohio/1/09
Cheshire/1/09
Bridgend/1/09
Herefordshire/1/09
Monmouthshire/1/09
Limerick/1/10
100
V223I
Lanarkshire/09
Dorset/09
Nottinghamshire/09
Oregon/1/10
California/2/10
Pennsylvania/6-15/11
Pennsylvania/3-7/11
Pennsylvania/2-5/11
Pennsylvania/5-5/11
Yokohama/AQ29/11
Yokohama/AQ5/11
Yokohama/AQ79/11
Yokohama/AQ53/11
100
Kentucky/1/11
Dubai/1/12
Dubai/2/12
Dubai/3/12
New York/1/11
Kentucky/4/11
100
Texas/12
Kentucky/1/12
Rastatt/1/12
Kentucky/2/12
Kentucky/3/12
N188T
PRE-DIV.
EURASIAN
AMERICAN
FLORIDA SUBLINEAGE CLADE 2
FLORIDA SUBLINEAGE CLADE 1

Figure 2

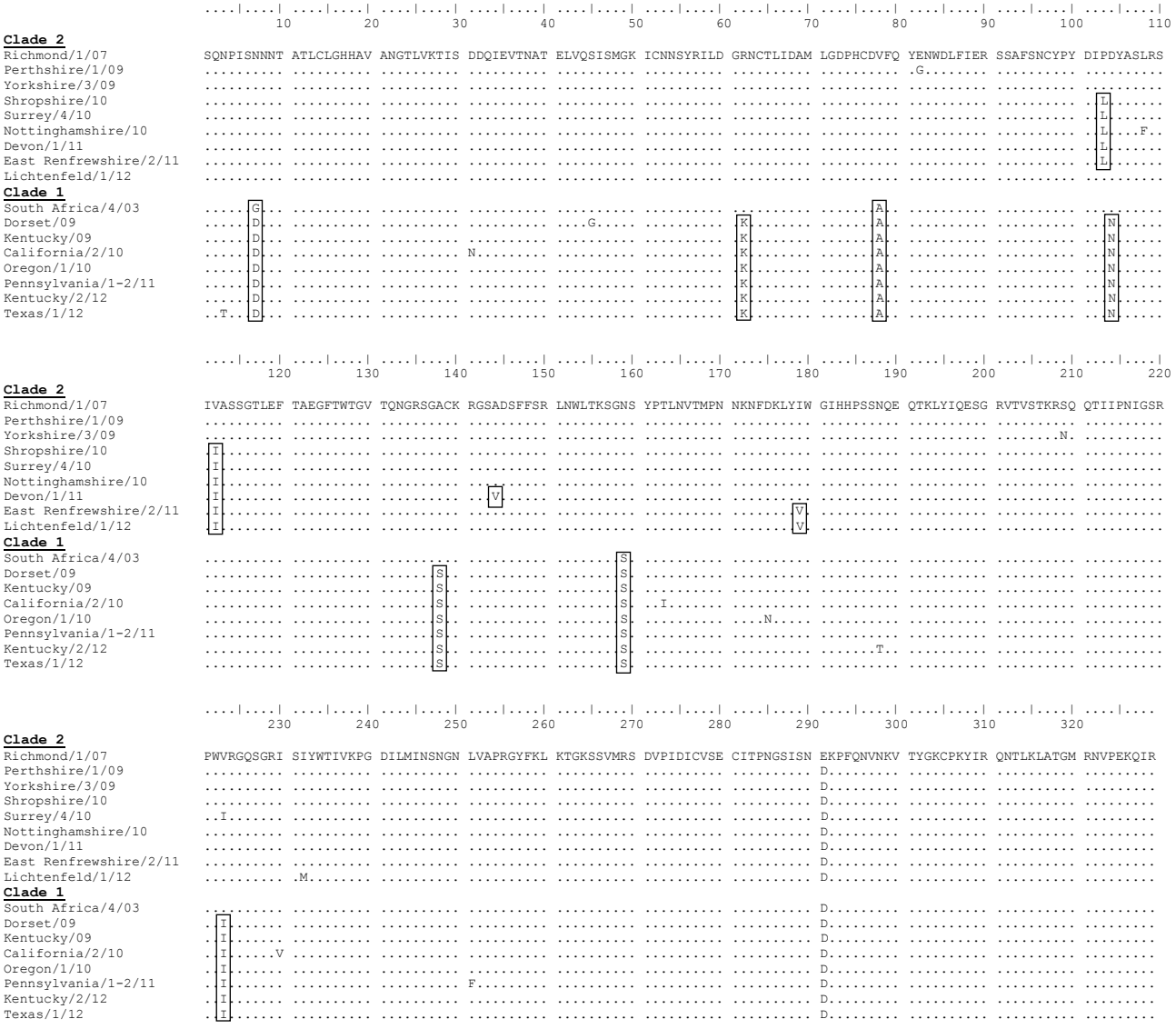
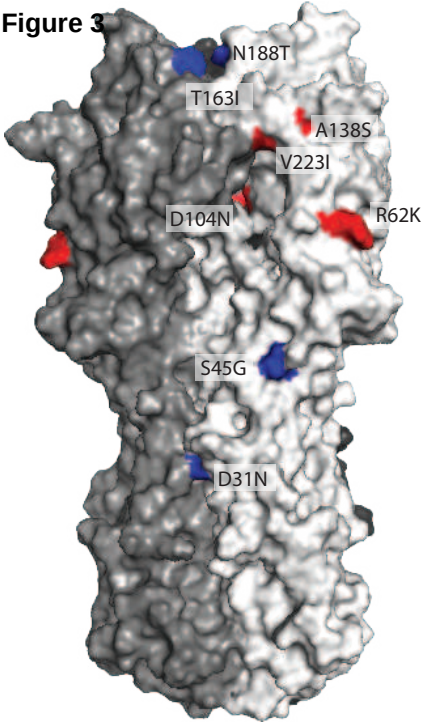
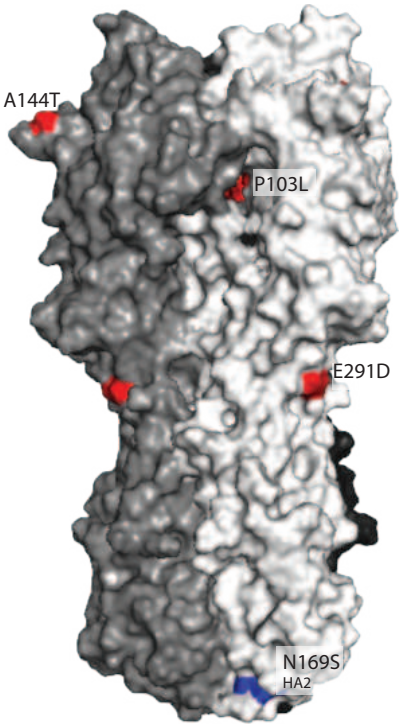

10 20 30 40 50 60 70 80 90 100 110
Clade 2
Richmond/1/07 SQNPISNNNT ATLCLGHHAV ANGTLVKTIS DDQIEVTNAT ELVQSISMGK ICNNSYRILD GRNCTLIDAM LGDPHCDVFQ YENWDLFIER SSAFSNCYPY DIPDYASLRS
Perthshire/1/09
Yorkshire/3/09
Shropshire/10
Surrey/4/10
Nottinghamshire/10
Devon/1/11
East Renfrewshire/2/11
Lichtenfeld/1/12
Clade 1
South Africa/4/03
Dorset/09
Kentucky/09
California/2/10
Oregon/1/10
Pennsylvania/1-2/11
Kentucky/2/12
Texas/1/12
120 130 140 150 160 170 180 190 200 210 220
Clade 2
Richmond/1/07 IVASSGTLEF TAEGFTWTGV TQNGRSGACK RGSADSFFSR LNWLTKSGNS YPTLNVTMPN NKNFDKLYIW GIHHPSSNQE QTKLYIQESG RVTVSTKRSQ QTIIPNIGSR
Perthshire/1/09
Yorkshire/3/09
Shropshire/10
Surrey/4/10
Nottinghamshire/10
Devon/1/11
East Renfrewshire/2/11
Lichtenfeld/1/12
Clade 1
South Africa/4/03
Dorset/09
Kentucky/09
California/2/10
Oregon/1/10
Pennsylvania/1-2/11
Kentucky/2/12
Texas/1/12
230 240 250 260 270 280 290 300 310 320
Clade 2
Richmond/1/07 PWVRGQSGRI SIYWTIVKPG DILMINSNGN LVAPRGYFKL KTGKSSVMRS DVPIDICVSE CITPNGSISN EKPFQNVNKV TYGKCPKYIR QNTLKLATGM RNVPEKQIR
Perthshire/1/09
Yorkshire/3/09
Shropshire/10
Surrey/4/10
Nottinghamshire/10
Devon/1/11
East Renfrewshire/2/11
Lichtenfeld/1/12
Clade 1
South Africa/4/03
Dorset/09
Kentucky/09
California/2/10
Oregon/1/10
Pennsylvania/1-2/11
Kentucky/2/12
Texas/1/12

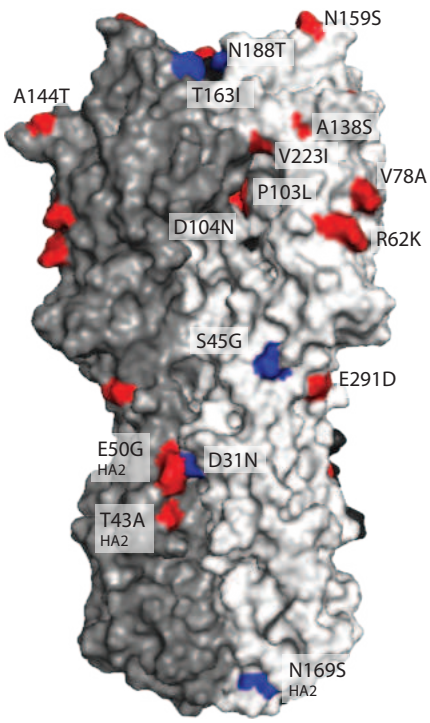
Figure 3



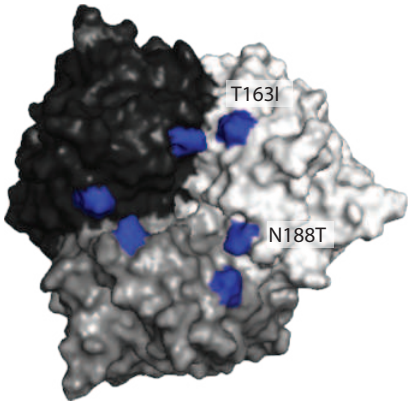
South Africa/4/03 v Clade 1

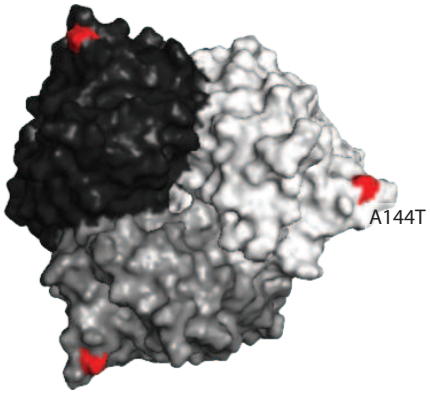


Richmond/07 v Clade 2



Clade 1 v Clade 2





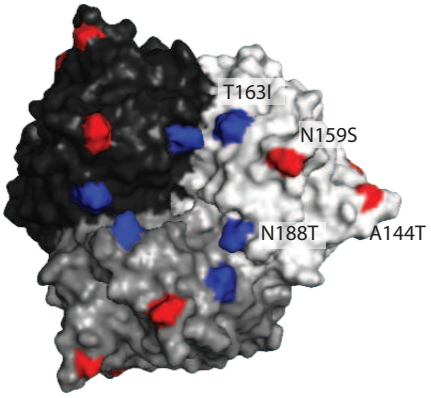


Figure 4

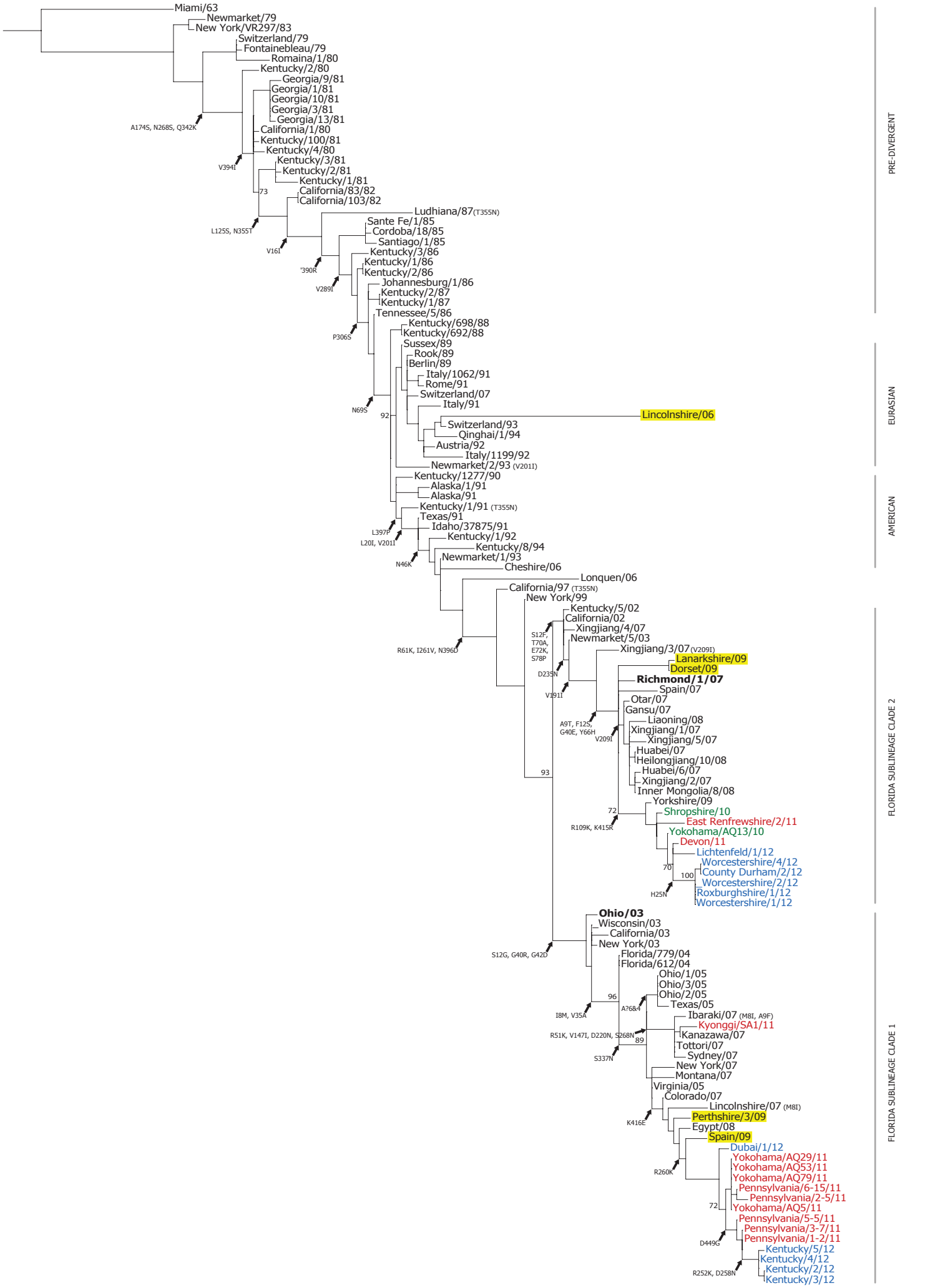

Miami/63
Newmarket/79
New York/VR297/83
Switzerland/79
Fontainebleau/79
Romaina/1/80
Kentucky/2/80
Georgia/9/81
Georgia/1/81
Georgia/10/81
Georgia/3/81
Georgia/13/81
California/1/80
Kentucky/100/81
Kentucky/4/80
Kentucky/3/81
Kentucky/2/81
Kentucky/1/81
California/83/82
California/103/82
Ludhiana/87(T355N)
Sante Fe/1/85
Cordoba/18/85
Santiago/1/85
Kentucky/3/86
Kentucky/1/86
Kentucky/2/86
Johannesburg/1/86
Kentucky/2/87
Kentucky/1/87
Tennessee/5/86
Kentucky/698/88
Kentucky/692/88
Sussex/89
Rook/89
Berlin/89
Italy/1062/91
Rome/91
Switzerland/07
Italy/91
Lincolnshire/06
Switzerland/93
Qinghai/1/94
Austria/92
Italy/1199/92
Newmarket/2/93 (V201I)
Kentucky/1277/90
Alaska/1/91
Alaska/91
Kentucky/1/91 (T355N)
Texas/91
Idaho/37875/91
Kentucky/1/92
Kentucky/8/94
Newmarket/1/93
Cheshire/06
Lonquen/06
California/97 (T355N)
New York/99
Kentucky/5/02
California/02
Xingjiang/4/07
Newmarket/5/03
Xingjiang/3/07(V209I)
Lanarkshire/09
Dorset/09
Richmond/1/07
Spain/07
Otar/07
Gansu/07
Liaoning/08
Xingjiang/1/07
Xingjiang/5/07
Huabei/07
Heilongjiang/10/08
Huabei/6/07
Xingjiang/2/07
Inner Mongolia/8/08
Yorkshire/09
Shropshire/10
East Renfrewshire/2/11
Yokohama/AQ13/10
Devon/11
Lichtenfeld/1/12
Worcestershire/4/12
County Durham/2/12
Worcestershire/2/12
Roxburghshire/1/12
Worcestershire/1/12
Ohio/03
Wisconsin/03
California/03
New York/03
Florida/779/04
Florida/612/04
Ohio/1/05
Ohio/3/05
Ohio/2/05
Texas/05
Ibaraki/07 (M8I, A9F)
Kyonggi/SA1/11
Kanazawa/07
Tottori/07
Sydney/07
New York/07
Montana/07
Virginia/05
Colorado/07
Lincolnshire/07 (M8I)
Perthshire/3/09
Egypt/08
Spain/09
Dubai/1/12
Yokohama/AQ29/11
Yokohama/AQ53/11
Yokohama/AQ79/11
Pennsylvania/6-15/11
Pennsylvania/2-5/11
Yokohama/AQ5/11
Pennsylvania/5-5/11
Pennsylvania/3-7/11
Pennsylvania/1-2/11
Kentucky/5/12
Kentucky/4/12
Kentucky/2/12
Kentucky/3/12
A174S, N268S, Q342K
V394I
73
L125S, N355T
V16I
'390R
V289I
P306S
N69S
92
L397P
L20I, V201I
N46K
R61K, I261V, N396D
S12F, T70A, E72K, S78P
D235N
V191I
A9T, F12S, G40E, Y66H
V209I
93
72
R109K, K415R
70
100
H25N
S12G, G40R, G42D
96
I8M, V35A
A76&4
R51K, V147I, D220N, S268N
89
S337N
K416E
R260K
72
D449G
R252K, D258N
PRE-DIVERGENT
EURASIAN
AMERICAN
FLORIDA SUBLINEAGE CLADE 2
FLORIDA SUBLINEAGE CLADE 1

Figure 5

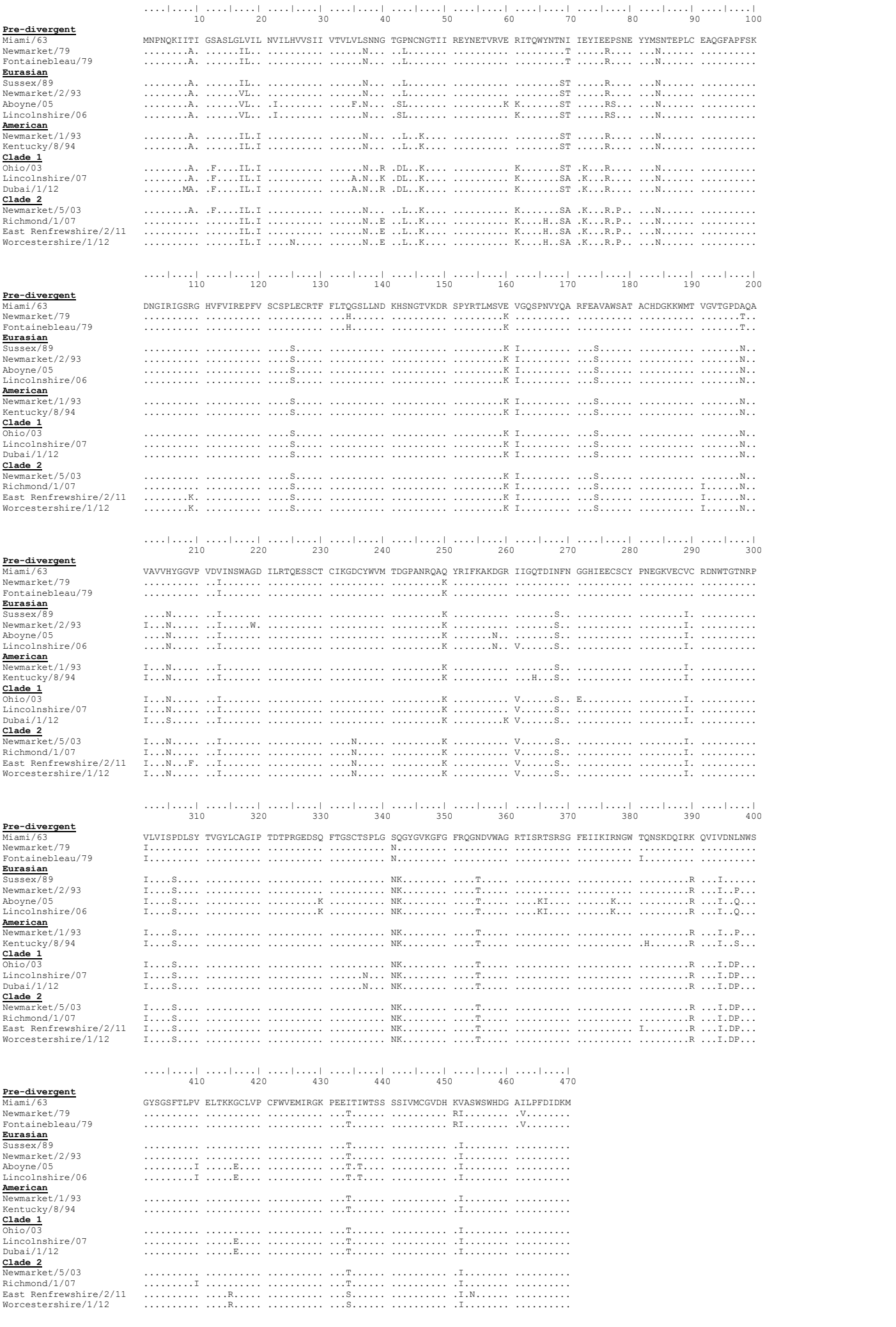
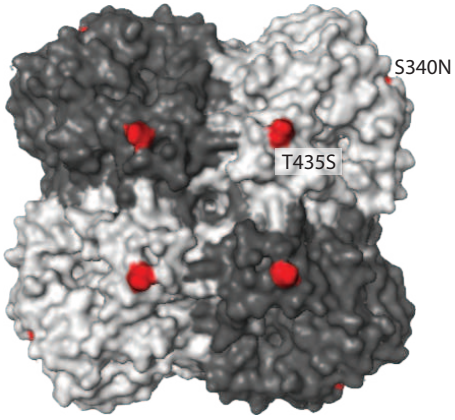
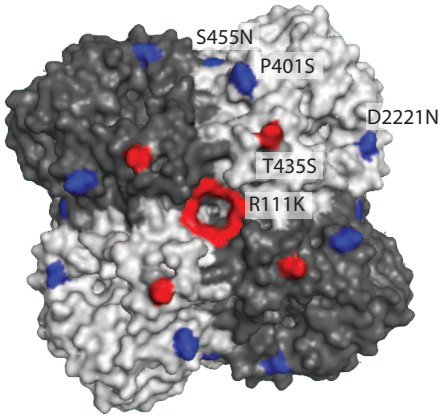

10 20 30 40 50 60 70 80 90 100
Pre-divergent
Miami/63 MNPNQKIITI GSASLGLVIL NVILHVVSII VTVLVLSNNG TGPNCNGTII REYNETVRVE RITQWYNTNI IEYIEEPSNE YYMSNTEPLC EAQGFAPFSK
Newmarket/79 ....A. ......IL.. ....N... ..L..... ....T .....R.... ...N...... ....
Fontainebleau/79 ....A. ......IL.. ....N... ..L..... ....T .....R.... ...N...... ....
Eurasian
Sussex/89 ....A. ......IL.. ....N... ..L..... ....ST .....R.... ...N...... ....
Newmarket/2/93 ....A. ......VL.. ....N... ..L..... ....ST .....R.... ...N...... ....
Aboyne/05 ....A. ......VL.. .I........ ....F.N... .SL..... ....K K.......ST .....RS... ...N...... ....
Lincolnshire/06 ....A. ......VL.. .I........ ......N... .SL..... .... K.......ST .....RS... ...N...... ....
American
Newmarket/1/93 ....A. ......IL.I ....N... ..L..K.... ....ST .....R.... ...N...... ....
Kentucky/8/94 ....A. ......IL.I ....N... ..L..K.... ....ST .....R.... ...N...... ....
Clade 1
Ohio/03 ....A. .F....IL.I ....N..R .DL..K.... .... K.......ST .K...R.... ...N...... ....
Lincolnshire/07 ....A. .F....IL.I ....A.N..K .DL..K.... .... K.......SA .K...R.... ...N...... ....
Dubai/1/12 ....MA. .F....IL.I ....A.N..R .DL..K.... .... K.......ST .K...R.... ...N...... ....
Clade 2
Newmarket/5/03 ....A. .F....IL.I ....N... ..L..K.... .... K.......SA .K...R.P.. ...N...... ....
Richmond/1/07 ....IL.I ....N..E ..L..K.... .... K....H..SA .K...R.P.. ...N...... ....
East Renfrewshire/2/11 ....IL.I ....N..E ..L..K.... .... K....H..SA .K...R.P.. ...N...... ....
Worcestershire/1/12 ....IL.I ....N..... ......N..E ..L..K.... .... K....H..SA .K...R.P.. ...N...... ....
110 120 130 140 150 160 170 180 190 200
Pre-divergent
Miami/63 DNGIRIGSRG HVFVIREPFV SCSPLECRTF FLTQGSLLND KHSNGTVKDR SPYRTLMSVE VGQSPNVYQA RFEAVAWSAT ACHDGKKWMT VGVTGPDAQA
Newmarket/79 ....H...... ....K ....T..
Fontainebleau/79 ....H...... ....K ....T..
Eurasian
Sussex/89 ....S..... ....K I......... ...S...... ....N..
Newmarket/2/93 ....S..... ....K I......... ...S...... ....N..
Aboyne/05 ....S..... ....K I......... ...S...... ....N..
Lincolnshire/06 ....S..... ....K I......... ...S...... ....N..
American
Newmarket/1/93 ....S..... ....K I......... ...S...... ....N..
Kentucky/8/94 ....S..... ....K I......... ...S...... ....N..
Clade 1
Ohio/03 ....S..... ....K I......... ...S...... ....N..
Lincolnshire/07 ....S..... ....K I......... ...S...... ....N..
Dubai/1/12 ....S..... ....K I......... ...S...... ....N..
Clade 2
Newmarket/5/03 ....S..... ....K I......... ...S...... ....N..
Richmond/1/07 ....S..... ....K I......... ...S...... .... I......N..
East Renfrewshire/2/11 ....K. ....S..... ....K I......... ...S...... .... I......N..
Worcestershire/1/12 ....K. ....S..... ....K I......... ...S...... .... I......N..
210 220 230 240 250 260 270 280 290 300
Pre-divergent
Miami/63 VAVVHYGGVP VDVINSWAGD ILRTQESSCT CIKGDCYWVM TDGPANRQAQ YRIFKAKDGR IIGQTDINFN GGHIEECSCY PNEGKVECVC RDNWTGTNRP
Newmarket/79 ....I..... ....K ....
Fontainebleau/79 ....I..... ....K ....
Eurasian
Sussex/89 ....N..... ..I..... ....K ....S.. ....I. ....
Newmarket/2/93 I...N..... ..I.....W. ....K ....S.. ....I. ....
Aboyne/05 ....N..... ..I..... ....K ....N.. ....S.. ....I. ....
Lincolnshire/06 ....N..... ..I..... ....K ....N.. V......S.. ....I. ....
American
Newmarket/1/93 I...N..... ..I..... ....K ....S.. ....I. ....
Kentucky/8/94 I...N..... ..I..... ....K ....H...S.. ....I. ....
Clade 1
Ohio/03 I...N..... ..I..... ....K .... V......S.. E......... ....I. ....
Lincolnshire/07 I...N..... ..I..... ....K .... V......S.. ....I. ....
Dubai/1/12 I...S..... ..I..... ....K ....K V......S.. ....I. ....
Clade 2
Newmarket/5/03 I...N..... ..I..... ....N..... ....K .... V......S.. ....I. ....
Richmond/1/07 I...N..... ..I..... ....N..... ....K .... V......S.. ....I. ....
East Renfrewshire/2/11 I...N...F. ..I..... ....N..... ....K .... V......S.. ....I. ....
Worcestershire/1/12 I...N..... ..I..... ....N..... ....K .... V......S.. ....I. ....
310 320 330 340 350 360 370 380 390 400
Pre-divergent
Miami/63 VLVISPDLSY TVGYLCAGIP TDTPRGEDSQ FTGSCTSPLG SQGYGVKGFG FRQGNDVWAG RTISRTSRSG FEIIKIRNGW TQNSKDQIRK QVIVDNLNWS
Newmarket/79 I......... .... N......... ....
Fontainebleau/79 I......... .... N......... .... I......... ....
Eurasian
Sussex/89 I....S.... .... NK........ ....T..... ....R ...I......
Newmarket/2/93 I....S.... .... NK........ ....T..... ....R ...I..P...
Aboyne/05 I....S.... ....K .... NK........ ....T..... ....KI.... ......K... ....R ...I..Q...
Lincolnshire/06 I....S.... ....K .... NK........ ....T..... ....KI.... ......K... ....R ...I..Q...
American
Newmarket/1/93 I....S.... .... NK........ ....T..... ....R ...I..P...
Kentucky/8/94 I....S.... .... NK........ ....T..... .... .H.......R ...I..S...
Clade 1
Ohio/03 I....S.... .... NK........ ....T..... ....R ...I.DP...
Lincolnshire/07 I....S.... ....N... NK........ ....T..... ....R ...I.DP...
Dubai/1/12 I....S.... ....N... NK........ ....T..... ....R ...I.DP...
Clade 2
Newmarket/5/03 I....S.... .... NK........ ....T..... ....R ...I.DP...
Richmond/1/07 I....S.... .... NK........ ....T..... ....R ...I.DP...
East Renfrewshire/2/11 I....S.... .... NK........ ....T..... .... I........R ...I.DP...
Worcestershire/1/12 I....S.... .... NK........ ....T..... ....R ...I.DP...
410 420 430 440 450 460 470
Pre-divergent
Miami/63 GYSGSFTLPV ELTKKGCLVP CFWVEMIRGK PEEITIWTSS SSIVMCGVDH KVASWSWHDG AILPFDIDKM
Newmarket/79 ....T...... .... RI......... .V........
Fontainebleau/79 ....T...... .... RI......... .V........
Eurasian
Sussex/89 ....T...... .... .I......... ....
Newmarket/2/93 ....T...... .... .I......... ....
Aboyne/05 ....I .....E.... ....T.T.... .... .I......... ....
Lincolnshire/06 ....I .....E.... ....T.T.... .... .I......... ....
American
Newmarket/1/93 ....T...... .... .I......... ....
Kentucky/8/94 ....T...... .... .I......... ....
Clade 1
Ohio/03 ....T...... .... .I......... ....
Lincolnshire/07 ....E.... ....T...... .... .I......... ....
Dubai/1/12 ....E.... ....T...... .... .I......... ....
Clade 2
Newmarket/5/03 ....T...... .... .I......... ....
Richmond/1/07 ....I ....T...... .... .I......... ....
East Renfrewshire/2/11 ....R..... ....S...... .... .I.N...... ....
Worcestershire/1/12 ....R..... ....S...... .... .I......... ....

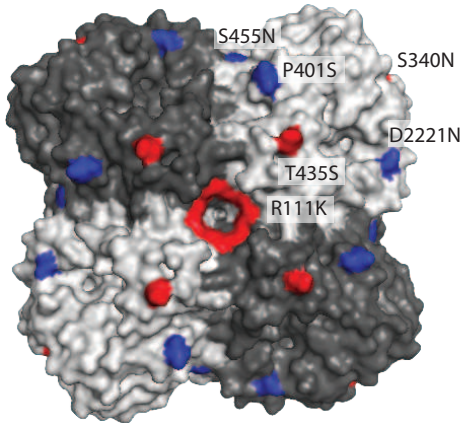
Figure 6



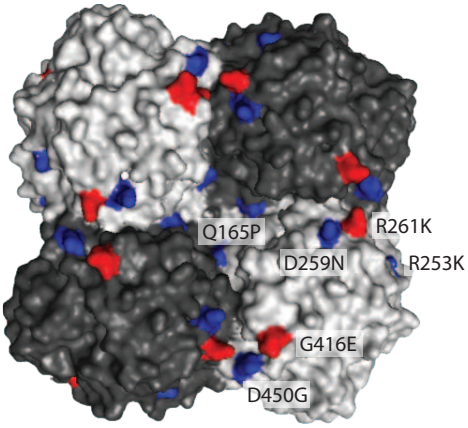
South Africa/4/03 v Clade 1

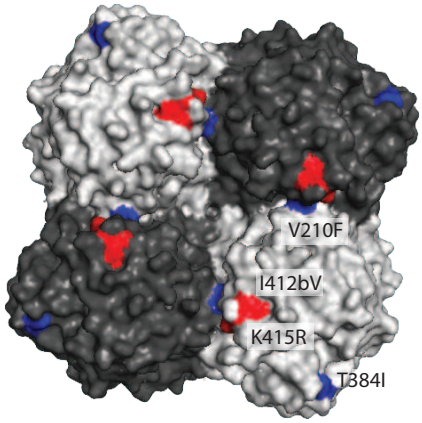


Richmond/07 v Clade 2



Clade 1 v Clade 2





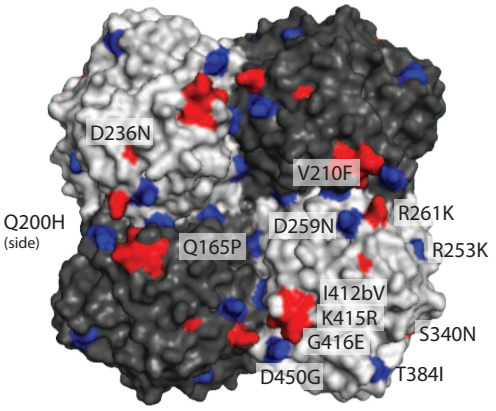


Figure 7

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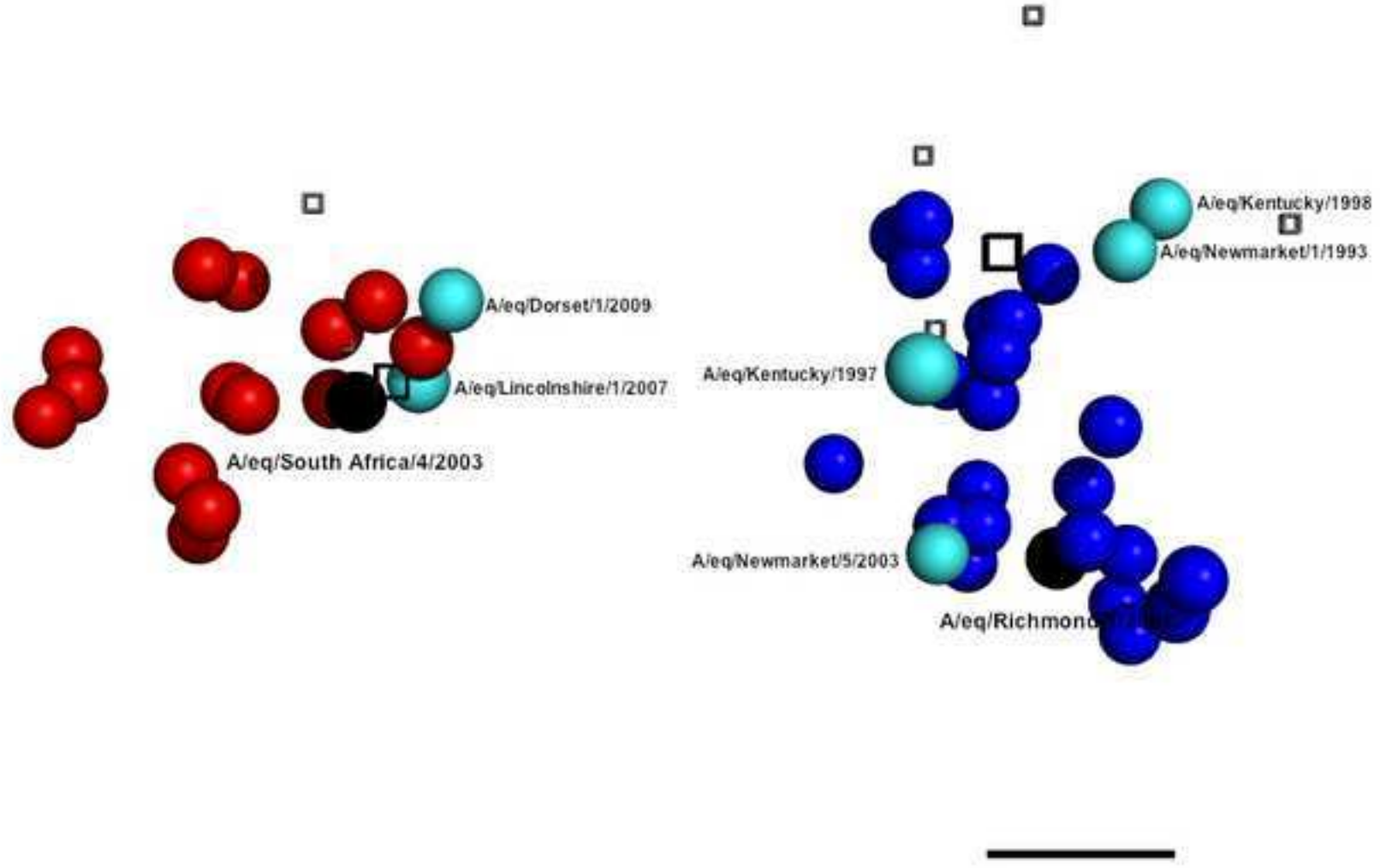

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